WEE1-inhibition and hypoxia

The impact of hypoxia and reoxygenation on the S-phase effects of the WEE1-inhibitor MK-1775

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WEE1-inhibition and hypoxia: *The impact of hypoxia and reoxygenation on the S-phase effects of the WEE1-inhibitor MK-1775*

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Abstract

The WEE1-inhibitor MK-1775 is currently in clinical trials as an anti-cancer drug. In response to DNA damage, human cells activate cell cycle checkpoints to temporarily halt cell cycle progression, and this helps facilitating DNA repair and is important to preserve genomic stability. It has been shown that inhibition of WEE1 leads to abrogation of the DNA-damage induced G2/M-checkpoint. Cancer cells often lack the p53-dependent G1/S-checkpoint, and may depend more on the G2/M-checkpoint for DNA damage repair than normal cells. The rationale behind using WEE1-inhibitors for cancer treatment is therefore that one can exploit this defective G1/S-checkpoint, in combination with G2/M-checkpoint abrogation, to make these cancer cells more sensitive towards DNA-damaging agents. However, recent studies have shown that in addition to regulating the G2/M-checkpoint, WEE1 also regulates normal S-phase progression in the absence of DNA-damaging agents. Previous work in our laboratory has shown that inhibiting WEE1 in normal S-phase causes DNA breakage and activates DNA damage signaling, and we believe that such S-phase damage also might contribute to cause cancer cell death following WEE1-inhibition.

Hypoxia is a common trait of solid tumors, and it develops due to rapid growth of cancer cells and insufficient growth of new blood vessels in the tumor, resulting in inadequate delivery of oxygen to the tumor cells. Tumor hypoxia is known to cause resistance to radiation therapy and certain chemotherapeutic drugs. An important issue regarding new cancer therapeutic drugs is therefore to investigate how tumor hypoxia influences the efficacy of the drug. As MK-1775 is already being tested in ongoing clinical trials, it is important to find out how the response to the drug might be altered when cancer cells are exposed to hypoxic conditions. Furthermore, previous work in our laboratory has shown increased S-phase damage in response to inhibitors of a related kinase, CHK1, following hypoxic exposure. In addition, several studies have shown that severe hypoxia can activate DNA damage signaling and replication stalling in S-phase cells. In relations to this, we wanted to investigate the impact of hypoxia on the S-phase effects of the WEE1-inhibitor MK-1775.

We performed experiments in U2OS osteosarcoma cells with several concentrations of MK-1775 at different levels of hypoxia. Our results showed no marked differences in the MK-1775-induced S-phase damage between normoxic cells and cells exposed to hypoxia. However, we found that the inhibitor is toxic to both hypoxic cells and in cells exposed to hypoxia followed by reoxygenation, demonstrating the potential for using MK-1775 for treatment of hypoxic tumors.

Contents

1 Introduction

1.1 General introduction

In 2010, 28271 people were diagnosed with cancer in Norway (www.kreftregisteret.no). People of all ages can get the disease, but the risk increases with age. Although an increasing percentage of people that get diagnosed with cancer survive (www.kreftregisteret.no), there is a need for more cancer-specific treatments as many of the current treatments have severe side effects. This has brought the focus onto the concept of personalized medicine – where treatment is tailored to exploit the genotypic and phenotypic traits of individual tumors, with maximal cancer cell destruction and minimal side effects. In order to achieve this, the treatment must target properties of the cancer cells that are not shared with normal healthy cells.

Two such properties are defects in the DNA damage response and hypoxia; both common traits of cancers that are usually not found in normal cells and tissues, and both contributors to genomic instability which is an important driver of carcinogenesis (Hanahan et al. 2011). Much research is now focused on exploring if and how these traits can be used to target cancer cells during treatment. Because cancer is a very heterogeneous disease, reflected in the variety of genotypic and phenotypic traits shown to occur in different cancers, this is not a straight forward process. Nevertheless, progress is made, and several therapeutic drugs targeting both the DNA damage response and hypoxia are currently used in the clinic or tested in clinical trials.

1.2 The DNA damage response

1.2.1 The DNA damage response – in general

To become cancerous, a cell must breach several barriers that normally work to maintain homeostasis and normal cell division, and accumulate a number of traits often referred to as hallmarks of cancer. Such hallmarks include sustaining proliferative signaling, resisting cell death and differentiation, and inducing angiogenesis (reviewed in Hanahan et al. 2000; Hanahan et al. 2011). Genomic instability is regarded an enabling characteristic of cancer, as it is a prerequisite for a cell being capable of acquiring such cancerous traits. Unrepaired DNA lesions can interfere with normal replication and transcription, and they can lead to mutations that might compromise cell viability or cause genomic instability (reviewed in Ciccia et al. 2010). To protect the cell from these possibly deleterious effects of DNA damage, a variety of signaling pathways and mechanisms that sense and repair DNA damage have evolved to preserve genetic integrity. Collectively these mechanisms are called the DNA damage response (DDR) (reviewed in Zhou et al. 2000; Ciccia et al. 2010).

There are a number of distinct but also interconnected signaling pathways and repair mechanisms that make up the DDR, reflecting the wide range of sources of DNA insults and the types of lesions they produce. Some DNA lesion types are directly repaired by protein-mediated reversal, however, most of the different pathways of the DDR consist of series of events, mediated by many different proteins (Jackson et al. 2009) (Figure 1). First of all, damage has to be discovered by so-called sensor proteins. There are a variety of sensors, again reflecting the large number of DNA lesion types. Secondly, signal transducers must relay information about the localization and types of lesions from the sensors to the effector proteins. This is done through signaling cascades, involving different posttranslational modifications like phosphorylations, ubiquitinations, poly(ADP-ribosylations) etc. (reviewed in Zhou et al. 2000; Ciccia et al. 2010). Downstream of the signal transducers are the effector proteins; a diverse group of proteins that are involved in repairing DNA lesions, controlling transcription and cell cycle progression, and inducing cell death or senescence if the damage is too severe to be repaired (Zhou et al. 2000) (see Figure 1). Other cellular responses might also be initiated, including chromatin remodeling, alterations in RNA processing and energy production, and replisome stability (Jackson et al. 2009).

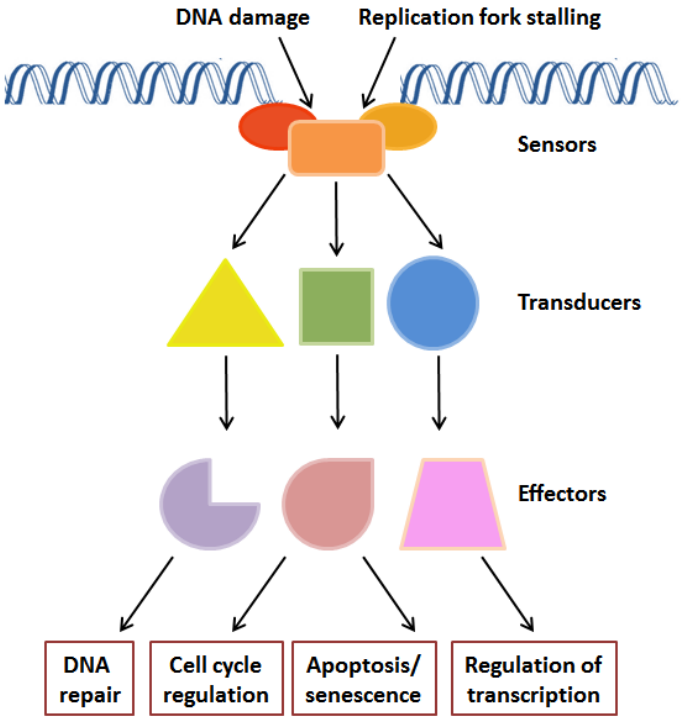

Figure 1: A schematic overview of the DNA damage response induced by DNA damage or replication fork stalling, and some of the cellular responses. Figure adapted from (Jackson et al. 2009).

1.2.2 Replication stress

Correct replication of the genome is essential for preserving genomic integrity. It is during the S-phase of the cell cycle that the DNA is duplicated, and at this time the genome is particularly vulnerable. Many DNA damaging agents can interfere with DNA replication, for instance by producing single strand breaks (SSBs) or double strand breaks (DSBs). Certain structural features of the chromosomes can also affect replication, like fragile sites and repetitive segments (reviewed in Errico et al. 2012).

DNA replication is initiated when origin recognition complexes (ORCs) bind to initiation sites distributed along the chromosomes, forming the pre-replicative complexes (pre-RCs). Following this, the central helicase complexes consisting of the mini-chromosome maintenance (MCM) 2-7 proteins, are loaded onto the chromosomes, making the origins licensed. It is then during S-phase that the Cyclin-dependent kinases (CDKs) together with other factors, convert the pre-RCs into pre-initiation complexes, which are able to unwind and replicate the DNA when they are joined by DNA-polymerases and components that

make up the active replicative helicase, for instance Cell division cycle 45 (CDC45) (reviewed in Takisawa et al. 2000; Ilves et al. 2010; Errico et al. 2012).

When encountering DNA lesions or other structures that interfere with replication progression, replication forks will arrest and this is called fork stalling. If this happens, completing DNA replication is dependent on stabilizing the stalled replication forks, followed by restart of replication when the problem is solved (Errico et al. 2012). In cases where replication forks stall, the arrested DNA polymerase will be uncoupled from the helicase complex, and the latter will continue to unwind the DNA (Zou et al. 2003). The resulting structural features of the DNA recruits components of the DDR, and this marks the coupling between replication stress and the damage response (Figure 1). The following activation of effector proteins leads to cellular responses like checkpoint induction, followed by slowing of DNA replication by inhibition of origin firing, and stabilization of replication forks (Nyberg et al. 2002; Petermann et al. 2010). This will be described in more detail below. If the DDR fails to discover or stabilize stalled replication forks, aberrant DNA structures forms and these are susceptible to attack by recombination proteins and/or nucleases. This leads to DSBs, which are severe lesions threatening the genomic integrity of the cell (Errico et al. 2012).

1.2.3 The ATM-CHK2 and ATR-CHK1 pathways of the DNA damage response

Two major signaling pathways activated by DNA damage or replication stress are the ATM-CHK2 and ATR-CHK1 pathways. The Ataxia telangiectasia mutated protein (ATM) and the Ataxia telangiectasia and Rad3-related protein (ATR) are PI3K-like kinases (PIKKs) recruited at early stages after DNA damage, and serve as signal transducers. They activate the effector proteins Checkpoint kinase 2 (CHK2) and Checkpoint kinase 1 (CHK1) respectively, as well as several other targets (reviewed in Ciccia et al. 2010). The cellular responses of these activations are diverse, and some of them will be described in this section.

The ATM-CHK2 pathway

DNA DSBs are the deadliest form of DNA lesions. When such lesions occur, the MRE11-RAD51-NBS1 (MRN) complex can bind to the site in conjunction with ATM (Thompson et al.

2012). At the damage site, ATM has several substrates. A major one of them is Histone H2AX, which is phosphorylated at S139, forming the DNA damage marker γH2AX (Stiff et al. 2004). Hundreds to thousands Histone H2AX proteins are phosphorylated on the chromatin around a double-strand break site, covering several megabases of chromatin (Rogakou et al. 1999; Pilch et al. 2003), and this creates a so-called focus, where many other proteins of the DDR are recruited and accumulate (Bonner et al. 2008; Kuo et al. 2008).

Another substrate of ATM is the effector kinase CHK2. CHK2 has several targets, associated with cell cycle regulation, apoptosis and transcription (reviewed in Smith et al. 2010). One of these targets is the transcription factor p53, an essential tumor suppressor protein involved in many damage response processes of which checkpoint regulation (described in more detail below) and apoptosis are important ones (reviewed in Mirzayans et al. 2012).

The cell has two main mechanisms for repairing DSBs; non-homologous end joining (NHEJ) and homologous recombination repair (HRR) (reviewed in Ciccia et al. 2010). NHEJ is a quick and error-prone repair mechanism, whilst HRR is much less error-prone as it uses a sister chromatid to serve as a template for the repair. HRR is therefore restricted to the S- and G2-phases of the cell cycle (reviewed in Yata et al. 2009). Additionally, initiation of HRR requires extensive DNA processing (reviewed in Ciccia et al. 2010). ATM is an important player in the preparation of DNA for HRR. Via ATM activation, the nuclease activity of Meiotic recombination 11 homolog A (MRE11) (a component of the MRN complex) is stimulated to generate a short segment of single-stranded DNA (Adams et al. 2006; Chen et al. 2008). These stretches can be extended by other nucleases to create larger segments of single-stranded DNA (ssDNA) that are subsequently coated with Replication protein A (RPA), and this serves as an activating template for both HRR and the ATR-CHK1 pathway (reviewed in Ciccia et al. 2010; Smith et al. 2010)

The ATR-CHK1 pathway

ATR is activated by recruitment to segments of ssDNA, together with its partner ATR-interacting protein (ATRIP) (Yang et al. 2006). ssDNA can occur when DNA replication is blocked and replication forks stall, or in response to DSBs as a result of strand resection. The generated ssDNA is coated with RPA, and the ATR partner ATRIP directly associates with this

protein-DNA complex (reviewed in Zou et al. 2003). ATR/ATRIP activation can in addition be mediated by nucleotide excision repair (NER), a repair mechanism where ssDNA is also generated. ATR has multiple targets, including Histone H2AX (ATR phosphorylates S139 on Histone H2AX like ATM, see above) and the effector kinase CHK1 (reviewed in Smith et al. 2010). Activated CHK1 has a number of substrates, both nuclear and cytoplasmic, which have several functions; they act in cell cycle regulation (described in more detail below), in HRR and in repression of transcription (reviewed in Bartek et al. 2003). As opposed to the ATM-CHK2 pathway, the ATR-CHK1 pathway is not only activated in response to DNA damage, but also functions during normal cell cycle, for instance in ensuring normal S-phase progression (Syljuasen et al. 2005).

1.2.4 DNA damage induced cell cycle checkpoints

Induction of cell cycle checkpoints is a cellular response to DNA damage or replication stress, mediated by components of the DDR. The cell cycle is divided into four phases, the first Gap (G1) phase, the Synthesis (S) phase, the second Gap (G2) phase, and the Mitotic (M) phase. The transition from one phase to the next is controlled by the ordered activation of Cyclin-dependent kinases (CDKs) that bind specific Cyclins to form active heterodimers (Figure 2A). The Cyclin/CDK complexes act in succession, activating multiple targets specific for the next cell cycle phase and thereby driving the cell cycle forward. Tight regulation of cell cycle transitions ensures that each process of one phase is complete before the cell enters the next phase (reviewed in Langerak et al. 2011).

When DNA damage occurs it is important that the cell does not go through with replication and/or division before the damage is repaired, in order to preserve genomic integrity. This is where the DNA-damage induced cell cycle checkpoints play a crucial role. There are three such checkpoints: the G1 to S-phase (G1/S) checkpoint, the intra S-phase checkpoint and the G2 to M-phase (G2/M) checkpoint. The way in which cell cycle progression is arrested can be direct, for instance through inhibition of CDK-activity, or indirect through transcriptional regulation (reviewed in Malumbres et al. 2009; Smith et al. 2010; Langerak et al. 2011).

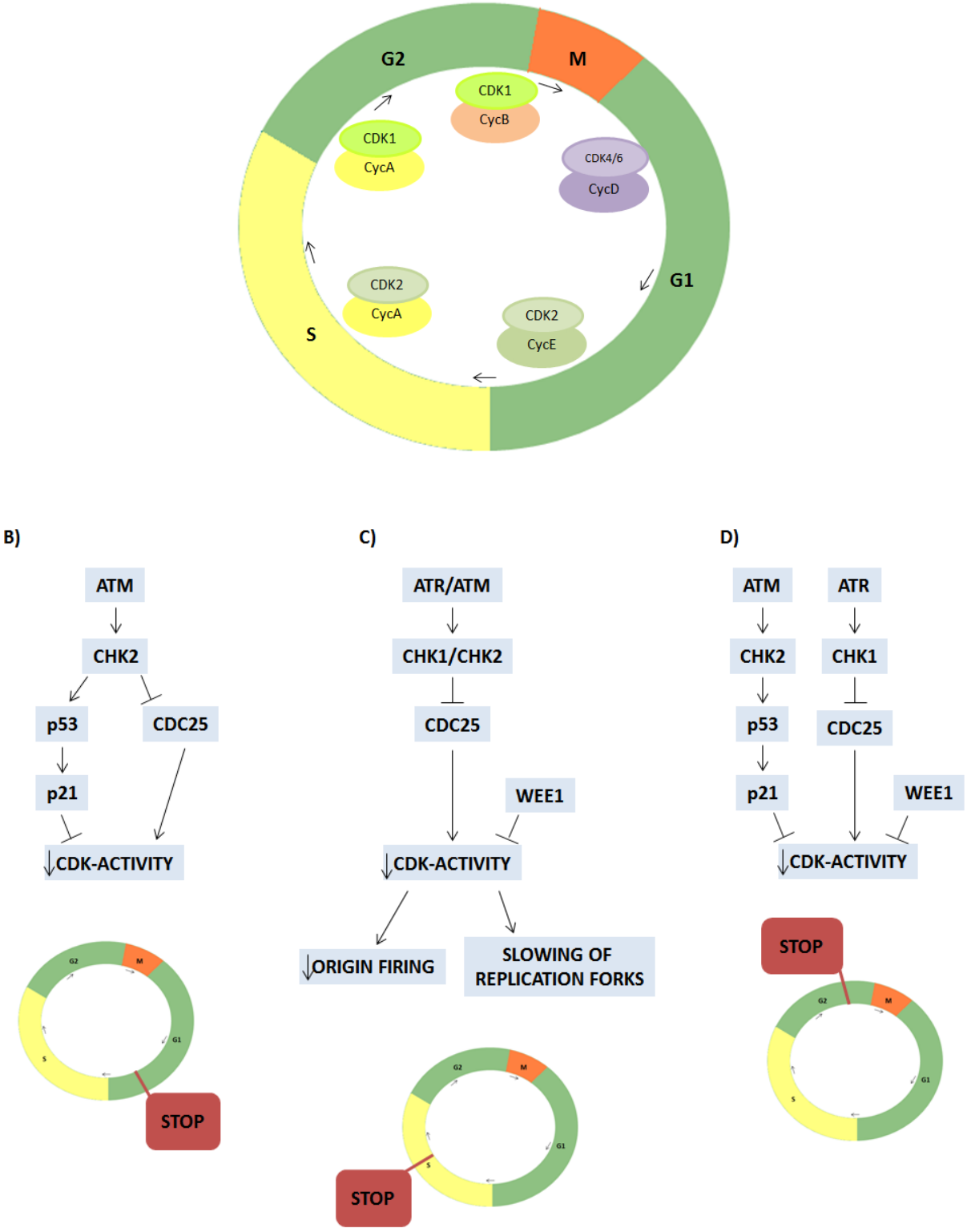

Figure 2: Schematic drawings illustrating DNA damage-induced checkpoint signaling. A) The cell cycle phases and the corresponding CDK-Cyclin complexes responsible for driving cell cycle progression. Modified from (Verbon et al. 2012). B) Induction of the G1/S-phase checkpoint. C) Induction of the intra S-phase checkpoint. D) Induction of the G2/M-phase checkpoint. B-D) Modified from (Kastan et al. 2004).

The G1/S-phase checkpoint

As mentioned previously, the ATM-CHK2 pathway responds to DNA DSBs. One target of CHK2 is the Cell division cycle 25 (CDC25) family of phosphatases (reviewed in Smith et al. 2010). These phosphatases remove certain inhibitory phosphorylations on Cyclin/CDK-complexes, allowing them to be activated. When DNA damage induces the DDR in G1-phase, the CHK2 protein can inhibit CDC25, thereby stopping the activity of Cyclin/CDK-complexes and inducing the G1/S-checkpoint (Figure 2B) (reviewed in Zhou et al. 2000; Bartek et al. 2001; Smith et al. 2010). However, G1/S-checkpoint maintenance is dependent on the function of the p53 transcription factor, which is activated in response to DNA damage by the ATM/CHK2-pathway (Figure 2B) (Kastan et al. 2004). One gene target of p53 is the *p21CIP/WAF1* gene, encoding the p21 protein which binds to and inhibits CyclinE/CDK2, a complex essential for S-phase entry. In addition, p21 inhibits S-phase entry by targeting the proliferating cell nuclear antigen (PCNA) (reviewed in Bartek et al. 2001; Mirzayans et al. 2012).

As p21 is transcriptionally up-regulated by p53, it takes some time for it to exert its full effect on checkpoint activation following DNA damage. So although the G1/S-checkpoint primarily depends on p53 and p21 activity, CHK2-mediated inhibition of CDC25 represents a more rapid way of inducing the checkpoint, as it does not act via transcription and protein synthesis, but directly through protein modifications. The p53-mediated checkpoint response on the other hand is essential to maintain the G1/S cell-cycle arrest until conditions are favorable for entering S-phase (reviewed in Bartek et al. 2001; Smith et al. 2010)

The intra S-phase checkpoint

As mentioned previously, the genome is especially vulnerable during S-phase. It is the ATR/ATM signaling machinery that controls the intra-S-phase checkpoint, of which the ATR-CHK1 pathway is the major contributor (reviewed in Smith et al. 2010) (Figure 2C). The ATM-CHK1 pathway induces the intra S-phase checkpoint in response to replication stalling, which among other things can occur when DNA lesions interfere with replication progression. This checkpoint involves inhibition of origin firing and slowing down of replication, as well as inducing replication fork stabilization (Bartek et al. 2001; Beck et al. 2010). When the ATR-

CHK1 pathway is activated, CHK1 will inhibit CDC25-activity, which leads to a decrease in CDK-activity (Sorensen et al. 2003). In addition, the WEE1 kinase can phosphorylate and inhibit the CyclinA/CDK2-complex (reviewed in Enders 2010). CDK-activity contributes to loading of CDC45 onto the chromatin, and this is a crucial step in initiating DNA replication (reviewed in Takisawa et al. 2000). Therefore, inhibition of this CDK-activity by CHK1- or WEE1 leads to a halt in additional origin firing (Figure 2C) (Zou et al. 1998; Feijoo et al. 2001). ATM-CHK2-mediated inhibition of CDC25 also plays a minor role in this checkpoint (Bartek et al. 2003).

As the intra S-phase checkpoint is initiated, it is essential to stabilize stalled replication forks to avoid components of the replication complex dissociating from the stalling site (fork collapse). This is crucial to complete DNA replication when conditions allow it, and to avoid recombination proteins and nucleases forming DSBs (see section 1.2.2). The ATR-CHK1 pathway is thought to be important during this step as well, for instance by keeping DNA polymerases at the stalled forks and by regulating HRR (reviewed in Errico et al. 2012).

Even though progression through S-phase is suppressed after checkpoint induction, the intra S-phase checkpoint does not induce cell cycle arrest, but a more transient delay of cell cycle progression. This is a result of the absence of a maintenance component, corresponding to p53/p21 during the G1/S-phase checkpoint. It has been suggested that prolonged S-phase arrest would have less efficient HRR because of limited amount of sister chromatids, and that it could increase the risk of already fired origins being fired again causing over-replication (Bartek et al. 2001; Errico et al. 2012).

The G2/M-phase checkpoint

CDK1 in complex with Cyclin B governs the transition from G2- to M-phase, and this complex is the target of the G2/M-checkpoint (reviewed in Kastan et al. 2004; Malumbres et al. 2009). Several of the regulators controlling the intra S-phase checkpoint are also central during G2/M-phase checkpoint activation. CHK1-mediated inhibition of CDC25 and WEE1-mediated inhibition of CDK-activity play central roles in arresting the cell cycle at this stage (Parker et al. 1992; Medema et al. 2012), to give time for DNA repair in cells that have not completed the repair during S-phase (Figure 2D).

Several mechanisms for maintaining the G2/M-checkpoint has been proposed. The p53-p21 pathway is important for G2/M-checkpoint maintenance (Bruno et al. 2006). Additionally, Breast cancer type 1 susceptibility protein (BRCA1), Breast cancer type 2 susceptibility protein (BRCA2) and or Partner and localizer of BRCA2 (PALB2) have been implicated, but the mechanisms behind this have not been elucidated (Xu et al. 2001; Menzel et al. 2011). Finally, DNA resection performed by the CtBP-interacting protein (CtIP) following DNA damage has also been shown to be required for sustaining the G2/M-checkpoint. Interestingly, this resection is not necessary for the ATR-CHK1 activation responsible for the immediate G2/M-checkpoint initiation (Kousholt et al. 2012).

1.2.5 Targeting the DNA damage response in cancer therapy

Components of the DDR are often lost or inactivated in cancers. As described above, this leads to genetic instability, which often results in accumulation of traits that drive cancer development. Therefore, cells with mutations affecting the DDR are often selected for during tumorigenesis. For instance, it has been shown that loss of the G1/S-phase checkpoint often happens early during cancer development (Syljuasen et al. 1999). However, there is another side to this story; loss of DDR pathways can also lead to increased cancer cell vulnerability.

When a tumor cell lacks one pathway of the DDR, it is often dependent on compensatory pathways for survival. This dependence can be exploited in cancer treatment based on the principle of synthetic lethality (Lord et al. 2012). In this context synthetic lethality means that targeting and inhibiting the compensatory pathway will render the cell unable to cope with endogenously occurring DNA damage. The resulting cell killing will then be tumor specific because normal cells still have the original DDR pathway, which can deal with the endogenous damage (Curtin 2012) (Figure 3). Another way of exploiting lost DDR pathways in tumor cells is to inhibit the compensatory pathway whilst simultaneously distributing a genotoxic agent. The consequence of this is that the cancer cells will be much more sensitive towards the genotoxic agent than normal cells. In addition, lower doses of the agent might be needed for cancer cell killing, and this is desirable when it comes to sparing normal tissues and minimizing side effects of treatment. This strategy is not based on synthetic

lethality *per se*, since a genotoxic agent is also added, but the principle can be referred to as synthetic sickness (Biss et al. 2012; Bouwman et al. 2012; Curtin 2012; Lord et al. 2012).

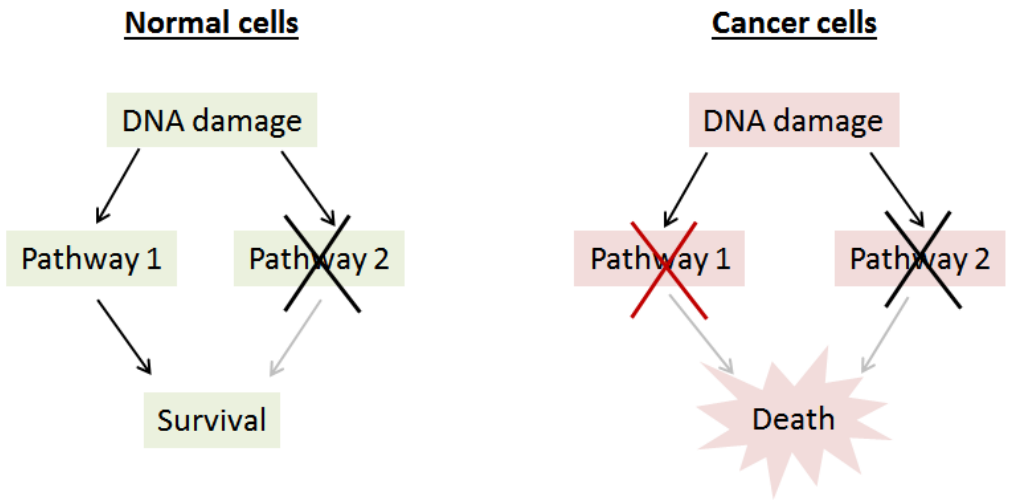

Figure 3: Schematic drawing illustrating the principle behind exploiting lost DNA damage response pathways in cancer treatment, to selectively target cancer cells. When targeting one DNA damage response pathway, a normal cell can survive DNA damage because it still has a compensatory pathway that repairs the damage. A cancer cell in which this compensatory pathway is lost will die because it will not be able to repair the damage. Modified from (Curtin 2012).

One well known example of using the principle of synthetic lethality when targeting the DDR in cancer therapy, is the use of poly(ADP-ribose) polymerase 1 (PARP1) or poly(ADP-ribose) polymerase 2 (PARP2) inhibitors in HRR-deficient tumors (reviewed in Ashworth 2008). The PARP proteins are some of the main components of base excision repair, a repair mechanism responsible for fixing a number of smaller DNA lesions such as SSBs. Several cancers have been shown to be defective in HRR, as a result of for example BRCA1 or BRCA2 mutations (the BRCA proteins are important components of the HRR machinery) (Bryant et al. 2005; Farmer et al. 2005). Such HRR-deficient cells can be killed when treated with PARP-inhibitors at concentrations that are non-toxic to normal cells with functioning HRR. The basis for this selective killing is suggested to be that endogenous levels of SSBs are not repaired when PARP is inhibited, and this results in stalled replication forks during DNA replication. In normal cells the stalled forks are resolved by HRR, but in the HRR-defective cancer cells these forks remain stalled or are incorrectly repaired – which leads to cell death (Bryant et al. 2005; Farmer et al. 2005).

An example of so-called synthetic sickness is combining genotoxic agents with drugs that cause cell cycle checkpoint abrogation. Loss of a checkpoint is a common feature of many cancers (Oren et al. 2010; Rivlin et al. 2011), and cells which lack a DNA damage induced checkpoint especially rely on the other checkpoints in case of DNA damage. Inducing DNA damage whilst simultaneously inhibiting factors responsible for the remaining checkpoints will then lead to the cancer cells dying because they go through with division with large amounts of unrepaired DNA lesions. Inhibitors of CHK1 and WEE1 are examples of checkpoint inhibitors currently in preclinical and clinical trials (Hirai et al. 2010; Chen et al. 2012; Kreahling et al. 2012), and this principle of targeting cancer cells is described in more detail below. Interestingly, inhibitors of such checkpoint inducers are also beginning to be explored as single agents. Oncogene activation in cancer cells often leads to increased replication stress followed by DNA damage and this can lead to synthetic lethality of checkpoint inhibitors in cells with activated oncogenes (Dai et al. 2010; Curtin 2012). This is an important point with regards to this master project, where the WEE1-inhibitor MK-1775 is used as a single agent.

1.3 The WEE1 kinase

1.3.1 WEE1: regulation and mode of action

Regulation of CDK-activity is important to ensure that the cell progress through the cell cycle in an ordered manner. WEE1 is well known as G2/M-checkpoint regulators it can phosphorylate and inactivate CDK1 (Lundgren et al. 1991). However, WEE1 is also important during normal cell cycle progression.

The WEE1 kinase consists of 646 amino acid residues, and it has an N-terminal regulatory domain, a central kinase domain and a short regulatory domain at the C-terminus (Igarashi et al. 1991; McGowan et al. 1995). It is a tyrosine kinase, responsible for adding a phosphate group on Y15 of CDK1 and CDK2 in complexes with Cyclins A, B or E. This is an inhibitory phosphorylation, blocking the enzymatic activities of the CDKs (Igarashi et al. 1991; McGowan et al. 1995; Perry et al. 2007). The activity of WEE1 is highest during S- and G2-phase, and it declines at mitotic entry. At this point, the inhibitory activity of WEE1 is

counteracted by several pathways (McGowan et al. 1995). Phosphorylation and subsequent degradation of WEE1 cause WEE1 protein levels to sink when the cell approaches mitosis (McGowan et al. 1995; Watanabe et al. 1995). Negative regulation of WEE1 can also be performed by several kinases, including polo-like kinase 1 (PLK1) and CDK1 itself. PLK1 and CDK1 phosphorylate WEE1 on residues S53 and S123 respectively, and these phosphorylations mark WEE1 for ubiquitin-mediated proteasomal degradation (Watanabe et al. 1995; Watanabe et al. 2004; Watanabe et al. 2005). As CDK1 also can phosphorylate and activate CDC25, there is a positive feedback-loop increasing CDK1 activity as the cell is entering mitosis (McGowan et al. 1995; Watanabe et al. 1995; Watanabe et al. 2004; Watanabe et al. 2005). There are also mechanisms preventing WEE1-degradation before mitosis, including autophosphorylation and the CDC14 phosphatase removing the Serine 123 phosphorylation added by CDK1 (Perry et al. 2007). In addition, it has been thought that WEE1 is positively regulated by binding to the 14-3-3 family of proteins (Lee et al. 2001), however this has been debated, and it is not fully clear how these proteins regulate WEE1-activity (reviewed in Perry et al. 2007).

While most studies have focused on WEE1 as a regulator of mitotic entry, more recently its role during S-phase has become the main focus. There are more origins of replication dispersed along the chromosome than is actually needed for replication. Such dormant origins can be fired when nearby replication forks stall (Woodward et al. 2006). Regulation of CDKs is important during normal S-phase progression to prevent too many origins being fired, and thereby preventing replication stress. This means that control of CDK-activity mediated by WEE1 (and CHK1) during DNA replication is essential for avoiding DNA damage and hereby maintaining genomic integrity (Beck et al. 2010; Fasulo et al. 2012; Sorensen et al. 2012).

1.3.2 S-phase damage following WEE1-inhibition

In addition to abrogation of the G2/M-checkpoint, inhibiting WEE1 causes S-phase damage (Beck et al. 2010). It has been shown that the increased CDK-activity caused by WEE1-inhibition leads to an increase in replication initiation. This is followed by nucleotide shortage which causes a reduction in replication fork speed and finally, this leads to DNA damage (Beck et al. 2012) (Figure 4). This DNA damage is thought to be caused by

unscheduled activity of the MUS81 structure-specific endonuclease. The studies underlying this theory show that co-depletion of MUS81 and WEE1-activity can abrogate most of the damage seen when only WEE1 is inhibited (Dominguez-Kelly et al. 2011; Beck et al. 2012) (Figure 4). However, exactly how WEE1 regulates MUS81-activity is not completely understood. The MUS81 nuclease normally cleaves branched DNA substrates, and plays important roles in generating temporary DSBs during HRR and replication restart following replication fork stalling (Hanada et al. 2007; Schwartz et al. 2012). One proposed mechanism for how WEE1-inhibition leads to DNA damage is that MUS81 cleaves stalled replication forks that occur after the CDK-activity is increased as a consequence of WEE1-inhibition (Dominguez-Kelly et al. 2011; Beck et al. 2012).

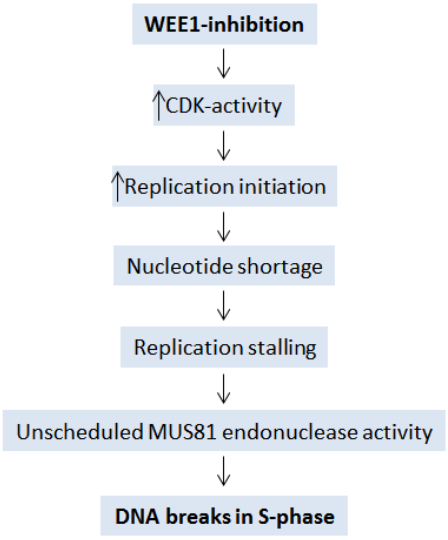

Figure 4: Model of how WEE1-inhibition causes S-phase damage. Modified from (Beck et al. 2012).

Interestingly, it was recently shown in yeast that positive regulation of MUS81 involves CDK-mediated phosphorylation (Gallo-Fernandez et al. 2012). This regulation causes the nuclease to be active only during a short period of the cell cycle (after replication, but before mitosis) when it resolves DNA intermediates that can remain after replication and that need to be resolved before mitosis. Furthermore it has been demonstrated (also in yeast) that premature activation of CDK-activity followed by Cdc5 (corresponding to PLK1)-dependent MUS81 activation causes incorrect replication and error-prone recombination repair (Szakal et al. 2013). It could therefore be possible that the increased CDK-activity following WEE1-

inhibition directly leads to unscheduled activation of MUS81 and subsequent DNA DSBs during S-phase. Moreover, a more direct regulation of MUS81-activity by WEE1 could be possible due to the fact that WEE1 and MUS81 are shown to physically interact (Dominguez-Kelly et al. 2011). Finally, it has been shown that forced activation of CDK1 through WEE1-inhibition leads to impaired HRR in interphase cells (Krajewska et al. 2012). Such insufficient repair might thus contribute to the damage seen after WEE1-inhibition.

CHK1 and WEE1 both inhibit CDK-activity in S- and G2-phase; CHK1 through inhibition of the CDC25 phosphatase and WEE1 through direct phosphorylation of CDKs (Perry et al. 2007). Like WEE1-inhibition, CHK1-inhibition results in increased CDK-activity, as well as increased origin firing and DNA damage in S-phase (Syljuasen et al. 2005; Scorah et al. 2009). A recent study showed that the MRE11 nuclease is required for the DNA damage occurring after CHK1-inhibition (Thompson et al. 2012). MRE11 was previously mentioned as a part of the MRN complex that localizes to DSBs at an early time point, and is a nuclease responsible for the initial processing of DSBs to single stranded DNA (Chen et al. 2008). It was proposed that MRE11 is the link between the unscheduled activities of CDC25 and CDK2 (caused by CHK1-inhibition), and the MUS81-induced DNA damage (Thompson et al. 2012). The ssDNA made by MRE11 was shown to be upstream of the DSBs, and in this way MRE11 may provide the substrate for MUS81 cleavage (Thompson et al. 2012). Whether the MRE11 nuclease is involved in causing DSBs in response to WEE1-inhibition, remains unknown.

1.3.3 WEE1-inhibition as an anti-cancer strategy

When it comes to targeting WEE1 in cancer therapy, it has mostly been focused on WEE1 as a regulator of the G2/M-checkpoint. Inhibiting WEE1 and thereby abrogating the G2/M-checkpoint in cancers already lacking the G1/S-checkpoint, has been shown to cause mitotic catastrophe when combined with conventional DNA-damaging drugs (Leijen et al. 2010; De Witt Hamer et al. 2011). More recent research has focused on the impact WEE1-inhibition can have on cancer cell survival even without additional genotoxic agents (Kreahling et al. 2012). Both of these strategies for cancer treatment are described in this section.

Mutations in p53 or the retinoblastoma protein (pRB) are very common traits in human cancers (reviewed in Levine 1997; Kawabe 2004), which leads to a malfunctioning G1/S-checkpoint. The lack of a functioning G1/S-checkpoint can increase the mutation rate in the

cell, which can drive carcinogenesis (Syljuasen et al. 1999). On the other hand, disruption of this checkpoint makes the cell more reliant on the other DNA damage induced checkpoints for survival following DNA damage. The reasoning behind using WEE1-inhibition together with genotoxic agents against cancers lacking the G1/S-checkpoint, is that abrogating the G2/M-checkpoint will render the cell unable to stop the cell cycle to give time to repair the induced damage, resulting in mitotic catastrophe (Kawabe 2004).

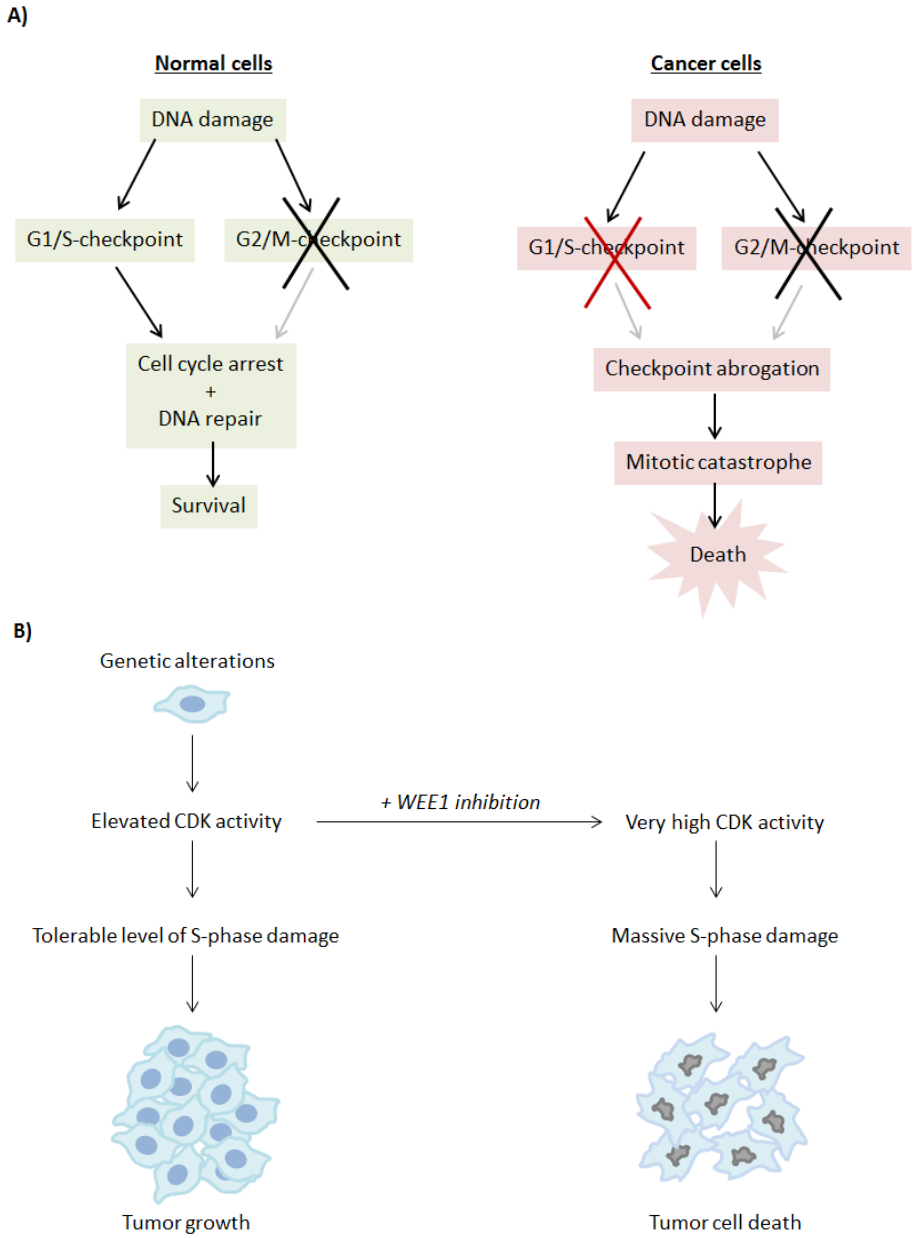

Figure 5: WEE1-inhibition causes cancer cell death by checkpoint abrogation and elevated CDK-activity. A) Abrogating the G2/M-checkpoint by WEE1-inhibition is not lethal to normal cells with a functioning G1/S-checkpoint, but leads to cell death in cells lacking this checkpoint. Modified from (Curtin 2012). B) Proposed model of how WEE1-inhibition can cause cancer cell death through elevated CDK-activity. Modified from (Sorensen et al. 2012).

Mitotic catastrophe occurs when the cell starts dividing with large amounts of unrepaired DNA lesions, leading to aberrant chromosome segregation and subsequent activation of the apoptotic pathway (Castedo et al. 2004; Kawabe 2004) (Figure 5A).

Several studies have used the small-molecule WEE1-inhibitor MK-1775 when analyzing WEE1-inhibition as a possible anti-cancer strategy for p53-defective tumors. They have shown that MK-1775 inhibits CDK1 phosphorylation(Y15), and that this abrogates the G2/M-checkpoint and causes mitotic catastrophe (Hirai et al. 2009; De Witt Hamer et al. 2011). MK-1775 can sensitize p53-defective tumor cells towards radiation and several DNA-damaging agents like 5-fluorouracil, gemcitabine, doxorubicin and pemetrexed (Hirai et al. 2009; Hirai et al. 2010; Bridges et al. 2011). Several of these DNA-damaging agents have different modes of action, and this shows that MK-1775 can be combined with different genotoxic agents to give decreased cancer cell viability.

Although most of the focus has been on inhibiting WEE1 in combination with other genotoxic agents, recent analyzes have shown that MK-1775 also has single-agent antitumor activity and cause significant cell death in sarcoma cell lines, independent on the cells p53 status (Kreahling et al. 2012). However, MK-1775 in combination with gemcitabine leads to an increased amount of cell death compared to MK-1775 alone. Interestingly, this sensitization has not been solely attributed to the G2/M-checkpoint abrogation; it has been proposed that MK-1775 causes abrogation of the S-phase arrest caused by gemcitabine, followed by forced mitotic entry leading to cell death (Kreahling et al. 2013).

As WEE1 has important functions in regulating S-phase, both during normal cell cycle progression and intra S-phase checkpoint initiation, it is not surprising that MK-1775 can have single agent activity. In cancer cells, replication stress is often seen as a result of oncogene activation and elevated CDK-activity (reviewed in Halazonetis et al. 2008). For cancer cells to survive the DNA damage caused by replication stress they need to make sure the levels of CDK-activity and subsequent DNA damage do not get too high. Regulators of CDKs like CHK1 and WEE1 are essential for this. Inhibiting for instance WEE1 can then cause the CDK-activity to become so high that the amount of the resulting DNA damage leads to cancer cell death (reviewed in Sorensen et al. 2012) (Figure 5B).

1.4 Hypoxia

1.4.1 Hypoxia in human tumors

Cancer cells, like normal cells, need blood supply for delivery of oxygen (O_2) and nutrients, and for removal of metabolic wastes. When tumor cells rapidly multiply, they therefore need to induce blood vessel growth to sustain the blood supply. As mentioned in the beginning of section 1.2, one hallmark of cancer cells is the ability to induce angiogenesis (the formation of new blood vessels). But angiogenesis takes some time, and this means that newly formed cells that are some distance away from blood vessels and out of the oxygen diffusion range (which is up to 200μm) will experience periods of reduced O_2 levels, a state known as hypoxia (reviewed in Hanahan et al. 2000; Brown et al. 2004; Bristow et al. 2008; Wilson et al. 2011). Furthermore, quickly formed new blood vessels in solid tumors often show structural abnormalities (Vaupel et al. 1989). This can lead to temporary obstructions of the vessels or variable blood flow in parts of the tumor, which also causes sub-populations of the cancer cells to experience hypoxia (Brown et al. 2004; Bristow et al. 2008).

Normal air contains 21% O_2 whilst normal body tissues have levels of O_2 ranging from 2% to 9%. What level of O_2 that is used to define hypoxia varies, but in this project hypoxia is regarded as O_2-levels below 2%. The levels of hypoxia can be divided into moderate hypoxia (0,1-2% O_2), severe hypoxia (0,01-0,1% O_2) and extreme hypoxia (<0,01% O_2). Furthermore, the time-range of hypoxia that cells experience can be divided into chronic hypoxia (lasting from hours to days), acute hypoxia (lasting from minutes to hours) and cycling hypoxia (cycles of hypoxia and reoxygenation) (reviewed in Ebbesen et al. 2004; Bertout et al. 2008). The sensitivity to hypoxia differs between different types of cells, but in the following sections a general outline of cellular reactions towards hypoxia is given.

The cellular responses to hypoxia are mediated by several pathways, the most significant being the hypoxia-inducible factor 1 (HIF1) mediated response (reviewed in Bertout et al. 2008). HIF1 is a transcription factor consisting of an α and a β subunit. At normal oxygen levels, HIF1α is hydroxylated by HIF-specific prolyl hydroxylases that are dependent on O_2, and subsequently marked for degradation by polyubiquitylation mediated by the von Hippel-Lindau (VHL) tumor suppressor protein. During hypoxia, this degradation ceases due to

inactivation of the prolyl hydroxylases, and HIF1α accumulates and translocates to the nucleus where it binds HIF-responsive elements (HREs) together with its binding partner HIF1β. This drives the expression of several proteins involved in the hypoxia-response. Other pathways involved in this response are the "unfolded protein response" (UPR) pathway and the "mammalian target of rapamycin" (mTOR) pathway. Together, these pathways mediate a wide range of cellular responses to hypoxia, including angiogenesis, metabolic adaption, autophagy, pH-regulation, repression of protein synthesis, and apoptosis (reviewed in Bertout et al. 2008; Wilson et al. 2011).

1.4.2 Hypoxia and the induction of the DNA damage response

Hypoxia and reoxygenation represents physiological stress for cells, and the DDR is activated at severe levels of hypoxia (<0,1% O_2) (reviewed in Olcina et al. 2010). In S-phase cells this activation is coupled to a rapidly induced replication arrest. It has recently been shown that the HIF subunit HIF1α negatively regulates the MCM complex and thereby inhibits origin firing and DNA replication (Hubbi et al. 2013). Furthermore, Ribonucleotide reductase (RNR) is an enzyme responsible for the production of nucleotides, and it is dependent on molecular oxygen for its function. Hence, during hypoxic conditions, nucleotide production is impaired and this leads to stalling of replication forks and accumulation of ssDNA followed by ATR activation (Chabes et al. 2003; Olcina et al. 2010; Pires et al. 2010). ATR in turn activates several targets, including γH2AX (Hammond et al. 2003) and CHK1. Interestingly, the purpose of CHK1 activation in response to hypoxia is not determined as it does not seem to be important for replication fork stabilization or inhibition of origin firing during hypoxic conditions (Hammond et al. 2004). Cells exposed to severe acute hypoxia remain replication competent, which means that they can resume replication after reoxygenation. However, S-phase cells exposed to severe chronic hypoxia cannot restart replication as stalled replication forks are destabilized (Seim et al. 2003; Pires et al. 2010).

In addition to the S-phase arrest seen in replicating cells exposed to hypoxia, hypoxic cells can experience a G1-phase arrest (Amellem et al. 1991).This arrest depends on the CDK inhibitors p27 and pRB. Whilst S-phase cells arrested during severe prolonged hypoxia cannot reenter the cell cycle after reoxygenation, cells arrested in G1-phase are able to do so (Amellem et al. 1996; Gardner et al. 2001). It has been proposed that the cells can avoid

possible damaging effects of initiating replication during hypoxia by stopping the progression through the cell cycle in G1-phase, and thereby remain replication competent (Amellem et al. 1991).

Hypoxic cells in tumors often regain blood supply, and are thereby reoxygenated. As opposed to hypoxia, reoxygenation has been shown to induce DNA damage through the production and subsequent actions of reactive oxygen species (ROS) (Freiberg et al. 2006). This DNA damage causes ATM to be activated and trigger a CHK2-dependent cell cycle checkpoint in G2-phase, as confirmed by decreased survival of CHK2-deficient cells after extreme hypoxia (<0,01% O2) followed by reoxygenation (Freiberg et al. 2006).

1.4.3 Hypoxia-induced genomic instability

Hypoxia is a part of the tumor microenvironment, and it is a common trait of solid tumors. By inducing genomic instability, hypoxia can affect the development of cancer (Hanahan et al. 2000; Hanahan et al. 2011).

There are two ways in which hypoxia can increase the mutation rate in tumor cells: (I) hypoxia followed by reoxygenation can directly lead to DNA damage, and (II) hypoxia induces inhibition of DNA repair (reviewed in Klein et al. 2010). DNA damage after reoxygenation is induced as a consequence of the activities of ROS, as mentioned in the previous section. Based on this it was proposed that cycling hypoxia acts as a mutagenic agent, increasing genetic instability in tumors (Hammond et al. 2003; Klein et al. 2010). Additionally, hypoxia has been shown to inhibit several DNA repair mechanisms. Nucleotide excision repair (NER) and mismatch repair (MMR) are both reduced by hypoxia; NER is inhibited on the enzymatic level and MMR is inhibited on the transcriptional level (Yuan et al. 2000; Mihaylova et al. 2003). Whilst these two repair mechanisms are involved in repairing smaller DNA lesions, one of the main repair mechanisms responsible for repairing DSBs, HRR, is also inhibited during hypoxia. It has been shown that HRR is decreased during moderate chronic hypoxia (0,2% O_2 for 72 hours) (Chan et al. 2008), severe acute hypoxia (0,02% O_2 for 16 hours) (Chan et al. 2010), and also for several days after reoxygenation following severe hypoxia (Bindra et al. 2004). DNA repair protein RAD51 homolog 1 (RAD51) and BRCA1 are critical components of HRR, and in response to severe hypoxia the transcription of the *RAD51* and *BRCA1* genes is repressed (Bindra et al. 2004; Bindra et al.

2005). As described previously, defects in the DDR compromises genomic integrity, and it is now recognized that hypoxia promotes genomic instability and as such represents a negative prognostic factor (Wilson et al. 2011).

1.4.4 Hypoxia as a negative prognostic and predictive factor

When it comes to cancer, a prognostic factor can give information about the effects that a characteristic (e.g. a tumor characteristic) can have on the patient outcome (Italiano 2011). As mentioned, hypoxia is said to be a negative prognostic factor for cancer patients, but this is not only due to it promoting genomic instability. One example of this is that low oxygen levels in tumors can lead to selection of cells with a more malignant phenotype. For instance, hypoxia provides a selective pressure in tumors for cells with decreased apoptotic potential. Hypoxia can induce cell death in transformed cells, but cells with p53 mutations or with overexpression of the apoptotic inhibitor protein B-cell lymphoma 2 (BCL-2) can overcome this hypoxia-induced apoptosis (Graeber et al. 1996). Cells with these characteristics are in that way selected for under hypoxic conditions, which results in an increasing number of the tumor cells being resistant to apoptosis. Another way in which hypoxia can serve as a negative prognostic factor is by enhancing the metastatic potential of tumor cells (reviewed in Rofstad 2000; Subarsky et al. 2003).

Whereas a prognostic factor can say something about the outcome of a disease based on certain characteristics, a predictive factor gives information about how a characteristic can affect the benefit from treatment (Italiano 2011). First of all, hypoxia serves as a negative predictive factor as the hypoxic cancer cells have poor blood supply and hence acquire smaller amounts of chemotherapeutic drugs that are administered via the vasculature (reviewed in Minchinton et al. 2006). Secondly, hypoxia is well-known to confer radiation resistance. Ionizing radiation (IR) used in cancer therapy kills cells by inducing DNA lesions, especially DSBs. In the absence of oxygen (at severe or extreme hypoxia), the fixation of DSBs is decreased, and hence the hypoxic cells are relatively radiation-resistance (reviewed in Brown et al. 2004; Bertout et al. 2008; Bristow et al. 2008). Finally, chemoresistance in hypoxic cells can be caused by several factors, including decreased drug efficacy at low O_2-levels, low effects of agents in hypoxic cells that have poor proliferation, or the up-regulation

of genes that encode drug pumps; membrane proteins that can actively pump many drugs out of the cell (reviewed in (Brown et al. 2004; Bristow et al. 2008).

1.4.5 Targeting hypoxic cells in cancer therapy

The impact of hypoxia on cancer treatment efficacy has led to development of treatment strategies targeting hypoxic cancer cells specifically. One example is the use of fractionated radiotherapy (where cells are given time to be reoxygenated and resensitized between radiation sessions) and also to the development of chemical radiosensitizers (chemicals that modulate radiosensitivity) and so-called bioreductive prodrugs (drugs that are activated specifically under hypoxic conditions) (reviewed in Overgaard 2007; Wardman 2007; Wilson et al. 2011).

Furthermore, although hypoxia often confers resistance to therapy, it sometimes might sensitize cancer cells to certain therapeutic agents. For instance, as it affects the DDR and induces genomic instability, several studies have been performed and are ongoing addressing whether or not hypoxia might increase the efficacy of inhibitors of the DDR. One example of this is the finding that decreased HRR during and following chronic hypoxia can resensitize cancer cells to IR and certain chemotherapeutic drugs (Chan et al. 2008). HRR-deficiency is selectively lethal with PARP-inhibitors (described in section 1.2.5*),* and increased clonogenic killing was demonstrated for hypoxic cells treated with such inhibitors (Chan et al. 2010). Another example comes from a recent study showing that reoxygenation sensitizes cells to CHK1-inhibition (Hasvold et al. 2013). This study found similar effects of CHK1-inhibition in hypoxic cells and normoxic cells. However, cells that had been exposed to prolonged hypoxia and subsequently reoxygenated showed decreased survival and increased S-phase damage compared to cells kept in normoxia. As CHK1-inhibition, similarly to WEE1-inhibition, leads to S-phase damage through unscheduled CDK-activity, we speculated that hypoxia/reoxygenation may also sensitize cells to the S-phase damage induced by WEE1-inhibition. This issue was addressed in this master project. Also, ATR-inhibition has been shown to increase cell sensitivity to hypoxia and reoxygenation (Hammond et al. 2004).

As described in this section, hypoxia can influence the efficacy of a wide range of anti-cancer agents. For this reason, testing the actions of new anti-cancer agents under hypoxic conditions would be important to give an insight into how they may work in a clinical setting.

2 Aim

The overall aim of this master project is to investigate whether hypoxia influences the DNA damage in S-phase cells caused by the WEE1-inhibitor MK-1775.

Specific aims of this project are:

(I) To find out if MK-1775 is effective in cells exposed to hypoxia

(II) To explore whether hypoxia has an impact on the S-phase damage following WEE1-inhibition

(III) To investigate if MK-1775-induced cell death is influenced by hypoxia.

3 Materials

3.1 Cell line and cell culturing

Material	Product	Supplier	Catalogue number
Medium	DMEM 1x with Glutamax	Life Technologies	31966-047
FBS	Fetal Bovine Serum, origin: South America	Life Technologies	10270-106, lot# 41G7121K
Antibiotic	Penicillin-Streptomycin, Liquid	Life Technologies	15140-122
PBS	1x PBS	Life Technologies	20012-068
Trypsin	Trypsin 0,25% with EDTA	Life Technologies	25200-056
WEE1-inhibitor	MK-1775	Axon	Axon 1494

3.2 Flow cytometry

Material	Product	Supplier	Catalogue number
PBS	10x PBS	Life Technologies	70011-051
Nonionic detergent	Igepal CA630	Sigma-Aldrich	13021
DNA dye	Hoechst 33258		
Primary antibody	Anti-H2AX-phospho (S139), mouse (clone JBW301), 1:500	Millipore	05-636
Primary antibody	Anti-Histone H3-phospho (S10), rabbit, 1:500	Millipore	06-570
Secondary antibody	Alexa Fluor® 647 goat Anti-mouse IgG, 1:500	Life Technologies	A-21235
Secondary antibody	Alexa Fluor® 488 donkey Anti-rabbit IgG, 1:500	Life Technologies	A-21206
EdU Kit	Click-iT® EdU Alexa Fluor 488® Flow Cytometry Assay Kit	Life Technologies	C35002
DNA dye	FxCycle Far Red Stain	Life Technologies	F10348
Flow tubes	BD Falcon 5ml polystyrene with cell strainer cap	VWR	734-0001

3.3 Immunofluorescence microscopy

Material	Product	Supplier	Catalogue number
Primary antibody	Anti-phospho-Histone H3 (S10), rabbit, 1:400	Millipore	06-570
Primary antibody	Anti-H2AX-phospho (S139), mouse (clone JBW301), 1:400	Millipore	05-636
Secondary antibody	Alexa Fluor® 568 donkey Anti-mouse IgG, 1:1000	Life Technologies	A-10037
Secondary antibody	Alexa Fluor® 488 donkey Anti-rabbit IgG, 1:1000	Life Technologies	A-21206
DNA dye	VECTASHIELD Mounting medium with DAPI	VECTOR Laboratories	H-1200
Coverslips	Coverslips 0,17+/-0,01mm, 12mm diameter	DNR Sentrallager	1014/2
Glass slides	Glass slides, 76X26mm	DNR Sentrallager	RH21088

3.4 SDS-PAGE and Western Blot

Material	Product	Supplier	Catalogue number
PBS	10x PBS	Life Technologies	70011-051
Tween	10% Tween20	Bio-Rad	161-0781
Lane Marker Reducing Sample Buffer	Lane Marker Reducing Sample Buffer-5ml	VWR	PIER39000
Acrylamide gel	Mini-PROTEAN TGX Precast Gels 4-15%	Bio-Rad	456-1086
Acrylamide gel	PIERCE 4-20% precise protein gels, 15 wells	VWR	25244
Running buffer	Tris Glycine SDS buffer	Bio-Rad	161-0772
Running buffer	20x Tris/Hepes/SDS Buffer	VWR	PIER28368
Molecular weight standard	Rainbow Marker, 250ul	VWR	RPN800E
Ponceau S	Ponceau S 1L solution	Sigma	P7170-1L
Nitrocellulose membrane	Nitrocellulose Membrane, 0.45 µm, 30 cm x 3.5 m, 1 roll	Bio-Rad	162-0115
Protein concentration measurement kit	Micro BCA Protein Assay Kit	VWR	PIER23235
Primary Antibody	Anti-WEE1, rabbit, 1:1000	Cell Signaling	4936
Primary Antibody	Anti-MUS81, mouse, 1:400	Abcam	ab14387

Material	Product	Supplier	Catalogue number
Primary Antibody	Anti-phospho-CDK1/CDC2 (Y15), rabbit, 1:1000	Cell Signaling	9111
Primary Antibody	Anti-MRE11, mouse, 1:1000	Abcam	ab214
Primary Antibody	Anti-PLK1, mouse, 1:1000	Invitrogen	37-7000
Primary Antibody	Anti-RAD51, rabbit, 1:1000	Santa Cruz	sc-8349
Primary Antibody	Anti-Histone H4 pan, rabbit, 1:1500	Millipore	05-858
Primary Antibody	Anti-MCM7, mouse, fridge, 1:200	Santa Cruz	sc-65469
Primary Antibody	Anti-γTubulin, rabbit, 1:3000	Sigma	T5192
Secondary Antibody	Horseradish peroxidase goat anti-rabbit IgG, 1:10000	Jackson ImmunoResearch	111-035-144
Secondary Antibody	Horseradish peroxidase donkey anti-mouse IgG, 1:10000	Jackson ImmunoResearch	715-035-150
ECL	SuperSignal® West Pico Chemiluminescent Substrate	VWR	PIER34080
ECL	SuperSignal® West dura extended duration	VWR	PIER34075
Film	Amersham hyperfilm ECL	VWR	28-9068-37

3.5 Clonogenic survival assay

Material	Product	Supplier	Catalogue number
Colony counter pen	eCount™, colony counter pen	VWR	710-0596
Methylene blue stain	Methylene blue-2-hydrat Before use: saturated Methylene blue diluted to 30% and 0,01% NaOH added	KEBOlab	1.1283-100

3.6 Buffers

Buffer	Content
Lysis buffer	2 % SDS 10mM TrisHCL, pH 7,5 + 100uM NO_3VO_4 added before use
Transfer buffer	900mL distilled H_2O 1,92M Glycine 250mM Tris Diluted 10x and 20% methanol added before use
Flow staining buffer	6,5mM Na_2HPO_4 1,5mM KH_2PO_4 2,7mM KCL 137mM NaCl 0,5mM EDTA 100µL Igepal added per 100mL buffer before use
Extraction buffer	0,5% TritonX-100 20mM Hepes, pH 7,4 50mM NaCl 3mM $MgCl_2$ 300mM Sucrose

4 Methods

4.1 Cell line, cell culturing and WEE1-inhibition

All experiments in this project were performed on U2OS cells, a human osteosarcoma cell line. This cell line has wild type genes encoding TP53 (Landers et al. 1997). However, the promoter of *CDKN2A*, encoding p16(INK4a) and p14(ARF) is methylated in this cell line (Park et al. 2002). The p16 and p14 proteins are involved in p53 stabilization through inhibition of Mouse double minute 2 homolog (MDM2) (Stott et al. 1998). p16 is also a direct inhibitor of CDK4/CDK6 (Sherr et al. 1995).

The U2OS cells were cultured in tissue culture flasks containing Dulbecco's Modified Eagle Medium (DMEM) complemented with 10% Fetal bovine serum (FBS), and 50 U/mL Penicillin Streptomycin. The cells were kept in a humidified incubator at 37°C and with 5% CO_2. Mycoplasma tests were performed regularly, and the cells have also been verified by short tandem repeat profiling.

In this project, WEE1-inhibition was obtained by using the small molecule inhibitor MK-1775 which inhibits WEE1-activity in an ATP-competitive manner and has an IC_{50} of 5,2nM (www.selleckchem.com). The chemical structure of MK-1775 is shown in Figure 6. The stock solution concentration was 100µM, and so diluting 1µL MK-1775 in 1mL DMEM gave a concentration of 100nM (we used concentrations in the range of 50nM to 300nM).

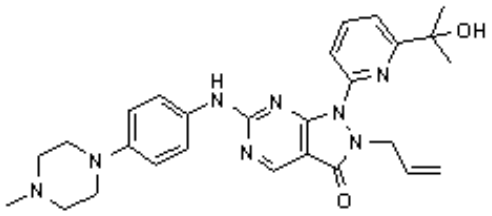

Figure 6: The chemical structure of the WEE1-inhibitor MK-1775 (http://www.axonmedchem.com).

4.2 Hypoxia treatments

In many of the experiments performed during this project, cells were exposed to low levels of oxygen (hypoxia). Hypoxic conditions were obtained using an InVivo$_2$ 200 hypoxia chamber (Ruskinn). This chamber functions as a normal cell incubator, keeping a temperature of 37°C and CO_2-level of 5%, but it is airtight. The chamber is equipped with a cuff and sleeve system for the arms for handling of samples, and an interlock for taking things in or out of the chamber without disrupting the hypoxic atmosphere. Flushing of N_2 into the chamber allows for the O_2 level to be reduced to 0,1 %. For further reduction of the oxygen levels, a mixture of H_2N_2 gas in combination with a palladium catalyst is used. The gas mixer in this hypoxia chamber measures the O_2 level every minute, and these measurements were checked after each experiment.

4.3 Flow Cytometry

4.3.1 General principles

A flow cytometer is an apparatus in which laser light of specific wavelengths is focused onto a fluid stream of single cells that pass through it. Several detectors measuring changes in the light properties (e.g. direction or wavelength) are placed where the stream of cells hit the light beam, and such changes are transformed into information about properties of the cell by the computer software.

The principle behind being able to analyze one cell at the time by flow cytometry is based on fluid mechanics. When a core stream of fluid (containing the cell sample) is injected into the center of a saline sheath stream in the cytometer, these two streams will not mix, and this condition is called laminar flow (Shapiro 2003, page 55). As the streams flow towards the area where the cell sample is exposed to light and the measurements are made, their areas are reduced; the core stream area becomes as small as 20µm (Shapiro 2003, page 56). In this way, only one cell will fit in the width of the stream.

When the light hits a cell, it will be deflected and change direction (scatter). Forward light scattering detection gives an estimate about cell size, whilst sideward light scatter detection

gives information about the inner complexities of the cell (for instance granularity) (Shapiro 2003, pages 4-5). In addition to light scatter detectors, a flow cytometer may have several fluorescence detectors. Fluorescent dye probes can be used for measuring numerous cellular properties, either by direct binding to a specific target in the cell or by indirect binding through antibody staining. When light of a certain wavelength hits such probes (collectively called fluorophores), the probe will absorb it and subsequently emit fluorescent light of a longer wavelength (lower energy). This shift in energy in absorbed versus emitted light is called the Stokes shift (Shapiro 2003, page 44). The emitted fluorescent light is measured by the fluorescence detectors in the flow cytometer and converted into information about a cell property, such as the amount of DNA or a specific protein. Since the machine can have several lasers sending out light of different wavelengths and several corresponding fluorophores can be used to stain the cell, several properties may be measured simultaneously. When using multiple fluorophores it is important that the emission spectrum of one does not overlap to a large extent with the absorption spectrum of another (Shapiro 2003, page 361). Such spectral overlap would influence the results, giving misleading information.

4.3.2 DNA profiles by Hoechst staining

Binding the minor groove of DNA, the fluorescent dye Hoechst can be used to measure the DNA content of cells. In our experiments we have used Hoechst 33258, which has an absorption maximum at about 350 nm and an emission maximum at about 461 nm.

DNA histograms in which the number of cells is plotted against the Hoechst signal can give information about how cells in a sample are distributed according to cell cycle phase (Figure 7A). Normal diploid cells in the G0- or G1-phase of the cell cycle are said to contain 2N DNA. Cells in G2- or M-phase will then contain 4N DNA, whilst cells with DNA contents between 2N and 4N are in S-phase.

However, if two or more cells are clumping together and are measured as a single event, the DNA histogram would deviate from the true cell cycle profile. For instance, a cell in G2- or M-phase will give off the same amount of fluorescence as two G0 or G1 cells (Shapiro 2003, page 290). Therefore, events arising from cell clumps need to be removed from the data set. In this project this was done by gating the cells based on the width versus area of the

Hoechst signal. This gating is based on the fact that it takes longer time for a doublet than a single cell to pass the laser beam, making the width of the signal larger for doublets than for single cells (Wersto et al. 2001), whilst the area (representing the amount of fluorescence given off) is the same (Figure 7B). Only single-cell events are included in the gate (Figure 7C; gate P1), and only these events are included in further analyses (see example of gated DNA profile in Figure 7D).

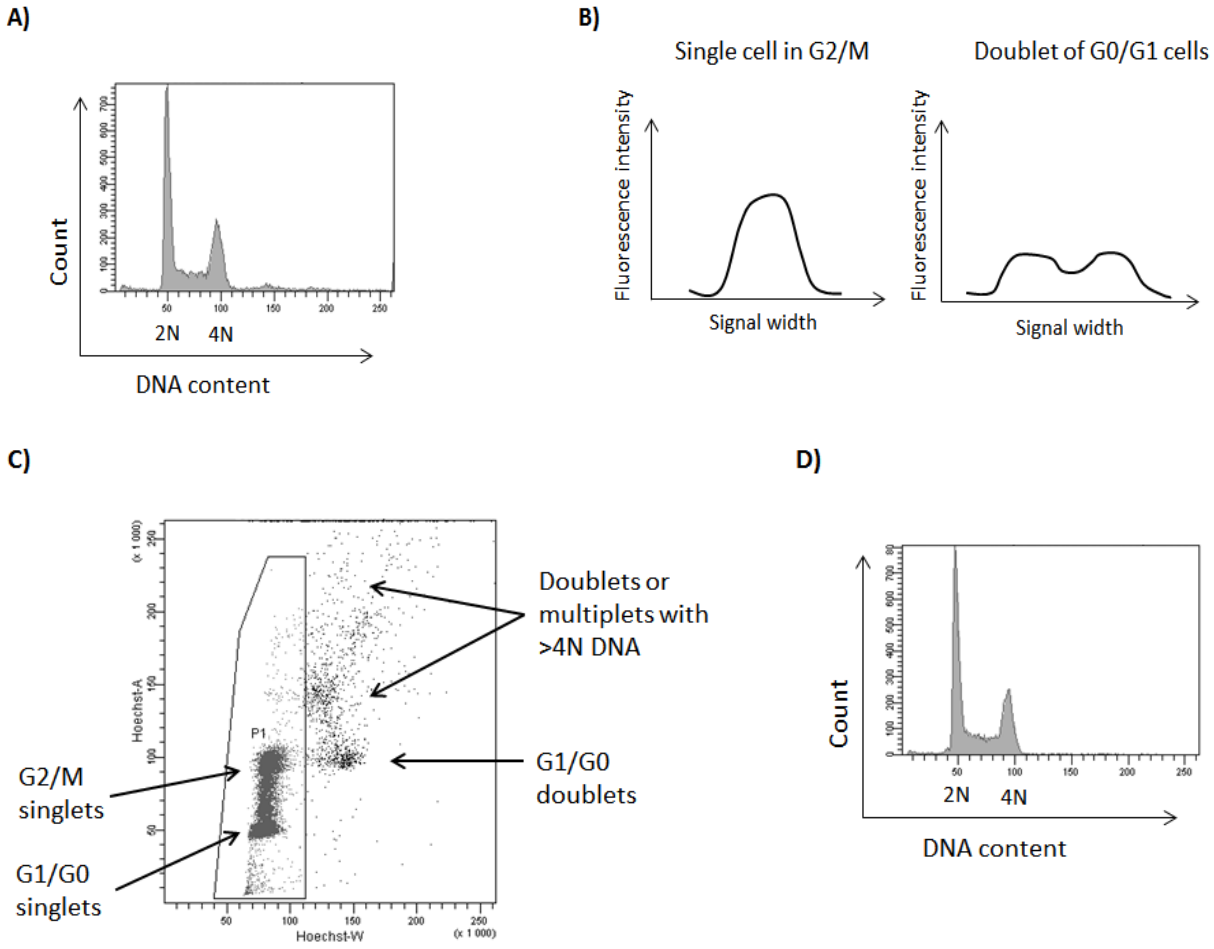

Figure 7: DNA profiles and gating based on Hoechst staining. A) Cell were stained with the DNA dye Hoechst 33258, and analyzed by flow cytometry. In the resulting DNA histogram (the cell cycle profile of the sample), the DNA content is indicated on the X-axis and the number of cells is indicated on the Y-axis. All recorded events are included. B) Schematic drawing illustrating how the signal width can differ whilst the area under the graph remains the same when the fluorescent signals of a single cell and a doublet are analyzed by flow cytometry. C) Dotplot from the same experiment as in A), where signal width is indicated on the X-axis and the amount of fluorescence given off (representing the amount of DNA) is indicated on the Y-axis. The gate shows how cell clumps are excluded from the data set. Only events in this gate are included in further analyses. D) DNA profile of the same sample as in A and C, but as opposed to the DNA profile in A, only events in the gate shown in C are included in this DNA profile.

4.3.3 Primary and secondary antibodies

For this project, samples analyzed by flow cytometry were stained with one or two primary antibodies. A rabbit antibody against phosphorylation of Histone H3 at the S10 residue (H3P) was used to identify mitotic cells as this phosphorylation can be used as a mitotic marker (Perez-Cadahia et al. 2009), while a mouse antibody against phosphorylation of Histone H2AX at the S139 residue (γH2AX) was used as a marker of DNA damage signaling (described in section 1.2.3). The secondary Alexa-fluorophore conjugated antibodies we used were Alexa Fluor® 488 (donkey anti-rabbit IgG, maximum absorption at 496 nm and maximum emission at 519 nm) and Alexa Fluor® 647 (goat anti-mouse IgG, maximum absorption at 650 nm and maximum emission at 665 nm) (www.lifetechnologies.com).

To check for possible background signals, caused by unspecific binding of secondary antibodies (Figure 8A), an untreated sample (only Hoechst staining) (Figure 8B), an untreated sample with secondary antibodies alone (no primary antibodies, only Hoechst staining) (Figure 8C) and an untreated sample stained with both primary (anti-H3P and anti-γH2AX) and secondary antibodies (Figure 8D) were analyzed and compared.

Figures 3B and 3C show that the gates (M, P2 and P3) are set high enough to avoid background signals that would influence our results. The gate M identifies mitotic cells (as it is placed where the cells show above background levels of fluorescence representing H3P, and contain 4N DNA). The gate P2 is set to catch all cells showing above background fluorescence representing γH2AX, whilst P3 only identifies cells having very high levels of γH2AX. These are the gates used in the flow cytometric experiments presented in this project.

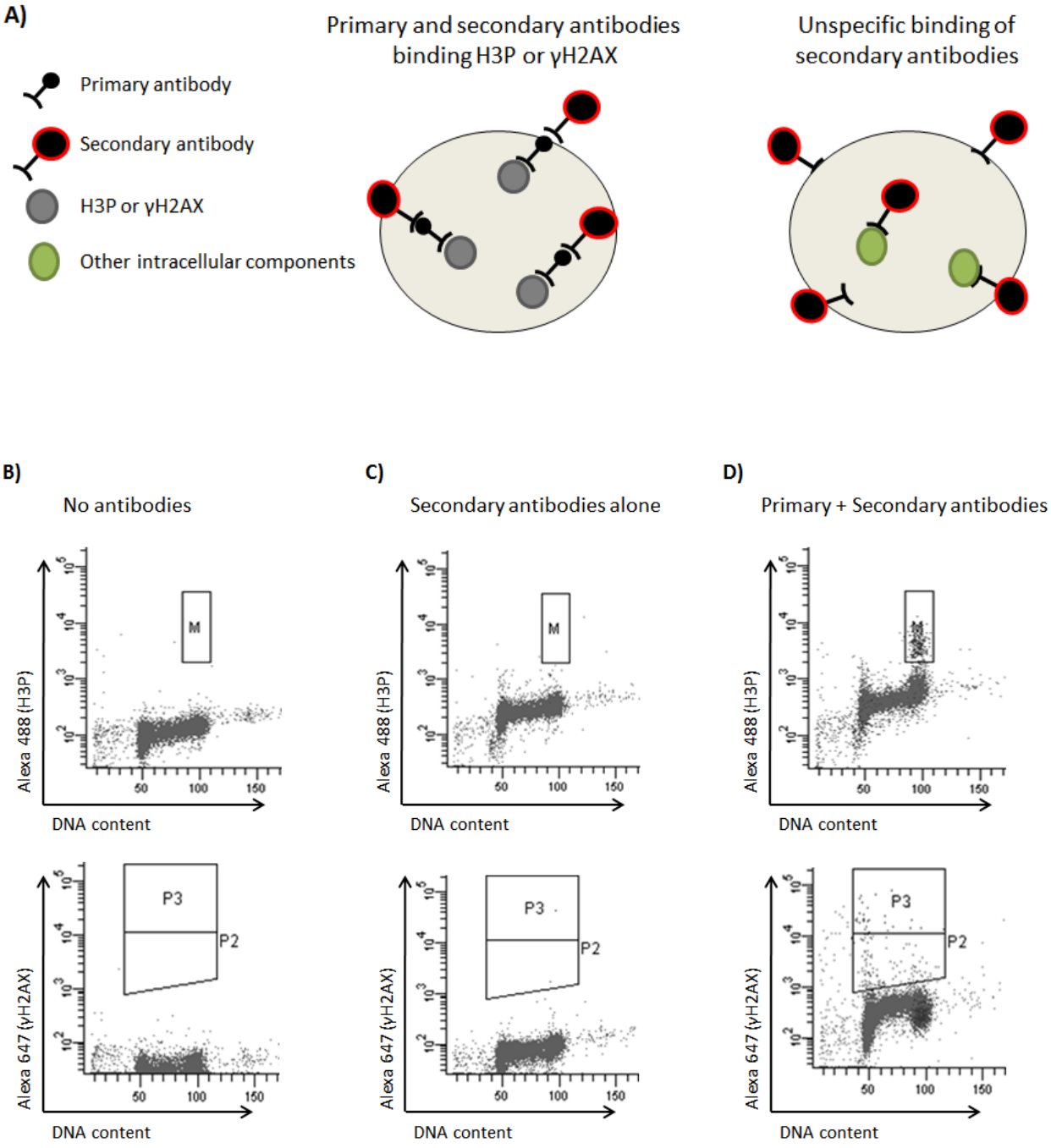

Figure 8: Checking for background signals. A) Schematic drawing illustrating how secondary antibodies might bind unspecifically to the cell membrane or intracellular components. B) Cells were stained with Hoechst only and analyzed by flow cytometry. The dotplots show measurements of signals from Alexa 488 (top dotplot) and Alexa 647 (bottom dotplot) as a function of DNA content. C) Cells were stained with Hoechst and the secondary antibodies Alexa 488 and Alexa 647. D) Cells were stained with Hoechst, primary antibodies against H3P and γH2AX, and the secondary antibodies Alexa 488 and Alexa 647. In this case the dotplots show the basal levels of H3P and γH2AX in the cells.

4.3.4 Sample preparation

After treatment the cells were trypsinated and transferred to 15 mL tubes together with the cell culture medium, then spun down (2000 rpm, 5 minutes). The supernatant was removed, and the cells fixed in ice cold 70% ethanol. These fixed cell samples were either prepared directly, or stored at -20°C. The next steps of sample preparation was adding Phosphate Buffer Saline (PBS) with 1% FBS to the sample tubes, spinning at 2000 rpm for 5 minutes, removing most of the PBS, spinning down again and removing the remaining PBS with a pipette. The resulting cell pellets were resuspended in 50µL of flow staining buffer containing 4% (w/v) non-fat milk and incubated for 5 minutes (blocking). This was followed by addition of primary antibodies diluted 1:250 in 50 µL flow staining buffer with milk, making the final antibody solution 1:500. The samples were incubated for 1 hour at room temperature, followed by washing in PBS with 1% FBS to remove unbound antibodies. After another round of centrifugation (2 times 5 minutes at 2000 rpm) the cell pellets were resuspended in 100 µL flow staining buffer with milk in which the secondary antibodies had been diluted 1:500. The samples were incubated for 30 minutes in the dark at room temperature. After another round of washing in PBS with 1% FBS and one final round of centrifugation (5 minutes at 2000 rpm), the cell pellet was resuspended in 0,5 mL Hoechst 33258 diluted 2,4 µL/mL in PBS (Hoechst stock solution was 624 µg/mL, final concentration 1,5 µg/mL). Finally the samples were transferred into BD Falcon flow tubes through a filter that removes cell aggregates (clumps). The tubes were stored in the dark at 4°C for at least 30 minutes before the samples were analyzed using a LSRII flow cytometer (BD Biosciences). Further data analyses were done using the BD FACSDiva software.

4.3.5 Using EdU to measure replication rates

During this project, studying the S-phase effects of the WEE1-inhibitor MK-1775 has been a major focus. Even though DNA staining gives information about the cellular DNA content and in this way identifies cells in S-phase, this does not say anything about to what extent an S-phase cell is actively replicating (actively synthesizing new DNA). To gather such information the thymidine analog EdU (5-ethynyl-2′-deoxyuridine) can be added to the cell medium a short time before trypsination and fixation of the cells. In actively replicating cells, EdU will

be incorporated into the newly synthesized DNA (Qu et al. 2011), and the extent of EdU uptake reflects the amount of replication that took place within the EdU labeling period (Beck et al. 2012). In our protocol 10µM EdU was added to the medium 1 hour before the cells were harvested and fixed as describe above. After washing the cells by adding PBS with 1% FBS to the sample tubes and spinning them twice at 2000 rpm for 5 minutes, as described above, 250 µL of Click-it™ (Invitrogen) reaction cocktail was used to resuspend the pellets. The fluorescent dye azide (Alexa Fluor® 488) in this cocktail binds EdU incorporated into DNA. Following incubation for 30 minutes in the dark at room temperature, the cells were again washed in PBS with 1% FBS, and spun twice at 2000 rpm for 5 minutes. Then, the cells were resuspended in 0,5 mL PBS containing 1 µL/mL FxCycle™ Far Red stain (DNA stain) and 10 µL/mL Ribonuclease A (degrades RNA). The FxCycle™ Far Red DNA stain has an absorption maximum at 640 nm and an emission maximum at 658 nm. The samples were kept at 4°C in the dark over-night before being analyzed in the flow cytometer. The median values of EdU uptake were determined using the BD FACSDiva software.

4.4 Immunofluorescence microscopy

Immunofluorescence (IF) microscopy has a lot in common with flow cytometric analyses with regard to the principles of fluorophores, light absorption and fluorescent light emission. In a fluorescence microscope, the light directed towards the cells is filtered by an excitation filter that only lets light of a certain wavelength pass (Shapiro 2003, page 8), and if fluorescent light is subsequently emitted by fluorophores in the samples, this is detected by fluorescence detectors. One microscope can have several excitation filters, specific for different wavelengths. Using multiple filters in combination with cell staining with different fluorophores makes it possible to examine several cell properties simultaneously. This can be very useful, for instance when investigating co-localization of cellular components.

In our experimental set-up 3-4 glass coverslips were placed at the bottom of the cell culture dishes before the cells were plated. After treatment, the coverslips (now having cells growing on them) were washed in PBS, and the cells were fixed by adding methanol/acetone (1:1) or 4% formaldehyde and leaving this on for 10-15 minutes at room temperature. If the purpose of the experiment was to look at chromatin-bound proteins, an extraction buffer

was added to the cells for 5 minutes prior to fixation to remove soluble proteins. After fixation, the coverslips were washed again in PBS, and cells were permeabilized in PBS containing 0,2% Triton X-100, a nonionic detergent, for 5 minutes. Following another step of washing with PBS, 150 µL of primary antibodies diluted in DMEM was added to the cells for incubation for 1 hour at room temperature. After washing off unbound antibodies using PBS, 150 µL of secondary antibodies diluted in DMEM was applied and left on for 30 minutes, also at room temperature. The secondary antibodies used in these experiments were Alexa Fluor® 488 and Alexa Fluor® 568 (maximum absorption at 578 nm and maximum emission at 603 nm). Unbound antibodies were washed off using PBS, and the coverslips were rinsed in distilled water and left to air-dry. Finally, as the coverslips were placed onto glass slides, one drop of DAPI was added to each of them. DAPI is a DNA dye with properties resembling Hoechst (Shapiro 2003, page 310). Its absorption maximum is at 358 nm and its emission maximum is at 461 nm. The coverslips were viewed in an Axio Imager Z1- microscope, using Axio Vision Release 4.8 software for image processing.

4.5 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a method used for separation of proteins according to their size. Polyacrylamide molecules in the gel form pores that the proteins migrate through when an electric current is applied. Small proteins will experience less resistance and migrate faster through these pores than large proteins.

Before starting the separation of proteins, the samples had to be prepared. In our set up, the cells were lysed with a lysis buffer containing 2% SDS, 10mM TrisHCl pH 7,4 and 100µM Sodium orthovanadate (Na_3VO_4), and they were boiled at 95°C for 5-10 minutes. Protein concentration measurements were performed using the Micro BCA Protein Assay Kit from Thermo Scientific. Lysate volumes were adjusted according to protein concentrations and lane marker reducing sample buffer (Thermo Scientific) was added. This buffer contains SDS, a denaturing detergent which binds to the proteins and gives them a uniform negative charge, Dithiothreitol (DTT) to reduce intra- and inter-molecular disulfide bonds, and glycerol to increase the density of the samples (so that they sink to the bottom of the wells)

in addition to a small-molecule indicator dye which allows observation of migration in the gel.

The gels used for this project had a polyacrylamide concentration of 4-20% or 4-15%, with the lowest concentration at the top of the gel and the highest concentration at the bottom. This allows for separation of proteins with a wide range of sizes as the low concentration of acrylamide is good for separating proteins of high molecular weight and the high concentration is best for separating smaller proteins (Walker 1984). The polyacrylamide gel was put in a BIO-RAD Mini PROTEAN^{TM} Tetra Cell chamber filled with a Tris/Glycine/SDS buffer (running buffer), and the samples were added to the wells of the gel. A molecular weight standard was applied to a lane for size reference. The gels were run at 100-120V for 1 hour. In response to the electrical current, the negatively charged proteins migrate through the gel towards the anode. At the end of the run, the smallest proteins will be located near the bottom of the gel and larger proteins that migrate slower will be located towards the top.

4.6 Western Blotting

After separation of the proteins by gel electrophoresis, they had to be transferred onto a membrane where the proteins of interest could be detected by immunoblotting. The gel and a nitrocellulose membrane were placed next to each other between filter paper and blotting pads, and this “sandwich” was put in a gel holder cassette. This cassette was then placed in a BIO-RAD Mini PROTEAN^{TM} II chamber filled with transfer buffer. The transfer buffer contains methanol and this helps the proteins being released from the gel (Pettegrew et al. 2009). In the chamber, the gel is oriented towards the cathode and the membrane towards the anode. By applying an electric current (100V in all experiments), the proteins are pulled out of the gel towards the anode, and onto the membrane. The transfer time was 50 minutes to 1 hour. The transferred protein bands on the membrane were visualized using Ponceau S staining (Salinovich et al. 1986). In combination with the visible molecular weight standard this allowed for the membrane to be cut into pieces according to the size of the proteins of interest. Blocking in PBS containing 10% Tween (PBST) and 5% milk was performed for one hour to prevent unspecific binding of antibodies to the membrane. Dilutions in PBST with 5%

milk of the primary antibodies specific for the proteins of interest were then applied and left on overnight at 4°C. The next day unbound primary antibodies were washed off using PBST, and dilutions of the secondary antibodies were applied and left on for one hour. After unbound secondary antibodies were washed off, Enhanced Chemiluminescent (ECL) solution was applied. The secondary antibodies used are conjugated to the enzyme Horseradish peroxidase (HRP), and this enzyme can catalyze a light-emitting reaction of the ECL solution that can be detected by a camera (we used the ChemiDoc from Bio-Rad) or on photographic film. The resulting images show bands where the primary antibodies bound the proteins on the membrane, and the molecular weight standard acts as a reference for whether or not a band represents a protein with the expected size (i.e. the protein of interest).

4.7 Clonogenic survival assay

Clonogenic survival assays are used to assess cell survival and ability to continue proliferation after certain treatments. In this project, cells were treated with the WEE1-inhibitor MK-1775 and hypoxia, and decreased cellular survival in response to MK-1775 in normoxia or hypoxia was investigated.

Before treatment, a certain number of cells (200-400) were plated in cell culture dishes with growth medium. Plating of cells was done less than 24 hours before treatment to minimize cell division events prior to treatment. The cells were plated in triplicates, i.e. three dishes were prepared for each treatment condition. After treatment, the medium was removed from the dishes, they were gently washed with PBS, and fresh growth medium was added. The dishes were incubated at 37°C and 5% CO_2 for 14 days, before fixation of the cells with 70% ethanol. Colonies were visualized by staining with Methylene blue solution. The dishes were then mixed and the colonies counted randomly (“blind”). One colony was defined to consist of at least 50 cells, which all originates from one of the originally plated cells (Franken et al. 2006). Plating efficiency was determined as the number of colonies counted relative to the original number of plated cells. Comparison of the plating efficiency in treated versus untreated samples gives information about the efficacy of the treatment. The surviving fraction of all the samples was determined as the plating efficiency of treated

normalized to untreated samples, and the mean survival fraction of each triplicate set was calculated.

5 Results

5.1 Initial experiments confirm previously described effects of MK-1775

The main focus of this study has been to investigate how hypoxia and reoxygenation influences the S-phase effects of the WEE1-inhibitor MK-1775 in U2OS osteosarcoma cells. The reasoning behind studying this is based on the results of many previous projects and published articles (see section 1). In order to confirm that the previously published effects important for this study also occurred under our experimental conditions, we first performed several initial experiments to check the effects of MK-1775 or hypoxia given as single treatments. These initial experiments were performed only one time each, and are presented in sections 5.1.1 to 5.1.3.

5.1.1 The WEE1-inhibitor MK-1775 accelerates mitotic entry

First, we wanted to confirm the effects of MK-1775 on the inhibitory phosphorylation of CDK1. The fact that WEE1 phosphorylates CDK1 on tyrosine 15 (Y15) and thereby inhibits CDK1 kinase activity (McGowan et al. 1995) is well-known, and in order to show that WEE1-inhibition reduces this phosphorylation we treated U2OS cells with MK-1775 and analyzed the cell protein contents using SDS-PAGE and Western blotting. The result presented in Figure 9A clearly shows a reduction in CDK1 phosphorylation in response to increasing concentrations of MK-1775.

As WEE1-activity prevents mitotic entry and induces the G2/M-phase cell cycle checkpoint (Parker et al. 1992), the next thing we wanted to confirm was increased entry into mitosis and G2/M-checkpoint abrogation in response to MK-1775. To do so, cells were treated with MK-1775 and analyzed by flow cytometry, and the mitotic fraction was identified using H3P antibody staining. To induce the G2/M-checkpoint, cells were exposed to ionizing radiation (IR) (Shackelford et al. 1999). Figure 9B (left graphs) shows an increase in the mitotic fraction after WEE1-inhibition, consistent with loss of CDK1 control. Furthermore, the G2/M-checkpoint induced by IR is diminished after MK-1775 inhibition, as there are few mitotic cells after IR exposure alone (Figure 9B, 3rd graph) whilst the number of mitotic cells after IR

exposure in combination with MK-1775 treatment (Figure 9B, last graph) is close to the number of mitotic cells after MK-1775 treatment alone.

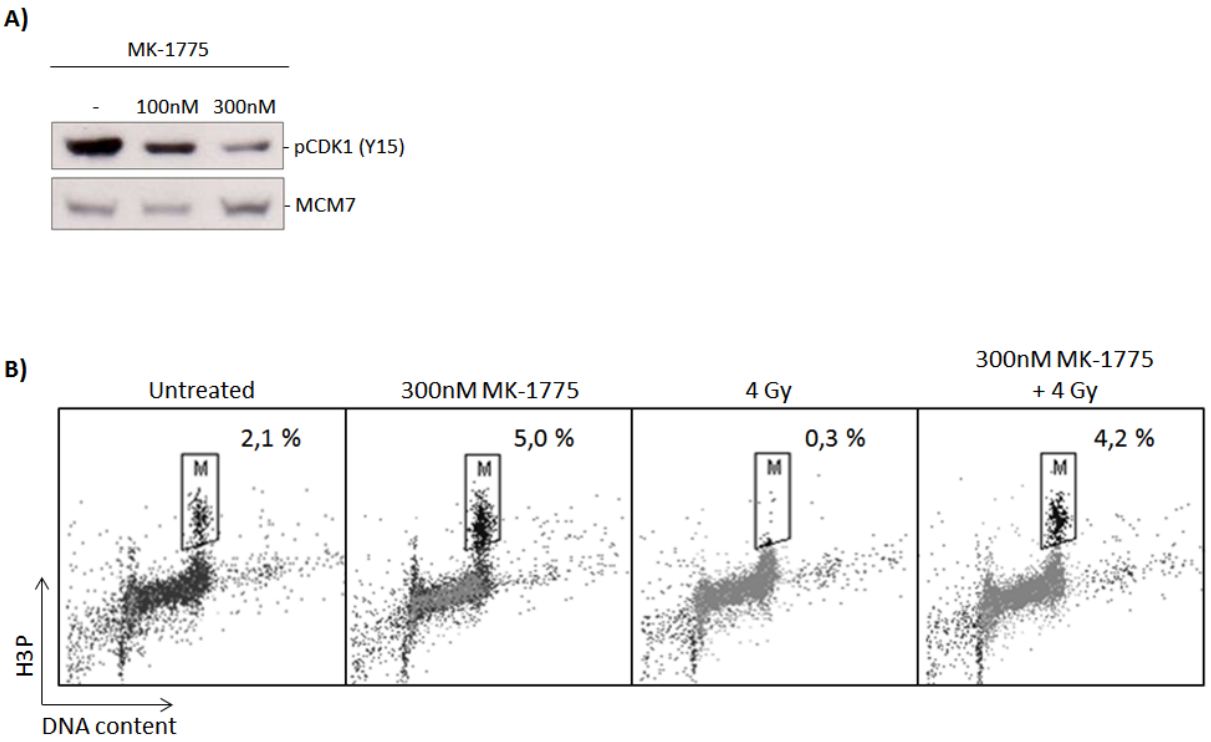

Figure 9: The effects of MK-1775 on CDK1 phosphorylation and mitotic entry. A) U2OS cells were treated with MK-1775 for 90 minutes, and cell protein contents were analyzed by SDS-page and Western blotting. MCM7 was used as a loading control. B) Flow cytometric analysis of U2OS cells treated with 300nM MK-1775 for 90 minutes either alone or in combination with exposure to 4 Gy of IR immediately after MK-1775 addition. Untreated cells were used as a control of mitotic fraction number, whilst cells exposed only to IR were used to show the G2/M-checkpoint induction. Mitotic fraction was identified using phosphor-H3 (H3P) antibodies, and the numbers of mitotic cells as a percentage of all events are indicated.

5.1.2 MK-1775 causes increased replication and S-phase damage

Next, we wanted to check that certain previously described S-phase effects of WEE1-inhibition also occurred under our experimental conditions. Increased replication initiation has been shown to be one consequence of WEE1-inhibition (Beck et al. 2010; Beck et al. 2012), and in order to demonstrate this, replication rates of MK-1775 treated cells were measured by giving a pulse of the thymidine analog EdU. The samples were analyzed by flow cytometry. The results, presented in Figure 10A, show an increase in the median value of EdU signals in response to increasing concentrations of MK-1775, indicating more replication

(due to additional origin firing) in the S-phase cells. After prolonged exposure to MK-1775 (Figure 10A, far right), replication is slightly decreased as compared to replication in cells with a shorter MK-1775 exposure time (Figure 10A, third graph). This is most likely a result of the replication stalling that occurs as too many replication origins are fired and the nucleotide pool is depleted in response to WEE1-inhibition (Beck et al. 2012).

As a consequence of the effects on replication initiation, WEE1-inhibition also causes S-phase damage (Beck et al. 2010; Dominguez-Kelly et al. 2011; Beck et al. 2012). To confirm that we get this S-phase damage after MK-1775 treatment, cells were exposed to the inhibitor, stained for the DNA damage-signaling marker γH2AX and analyzed by flow cytometry. The result presented in Figure 10B, right graph, distinctly show γH2AX signals being strong in the cells that have DNA contents between 2N and 4N and thus are in S-phase. We also wanted to visualize this damage using IF, and so cells were treated with the inhibitor, stained for both H3P (to mark mitotic cells) and γH2AX, and subsequently viewed in the microscope. A sample exposed to IR was used as a positive control for DNA damage. In Figure 10C we can clearly see the strong γH2AX staining caused by MK-1775 in some of the cells that are not in mitosis. Due to extraction being performed before fixation of the cells (in order to detect only chromatin-bound γH2AX) we could not co-stain with antibodies to common S-phase markers such as Cyclin A in these samples, but combining these results with the results presented in Figure 10B leads us to conclude that the γH2AX signals are mostly in S-phase cells. Some of the MK-1775 treated cells have pan-nuclear γH2AX staining. This is not seen in the IR-exposed cells, that all have distinct foci of γH2AX signals (Figure 10C, two bottom rows).

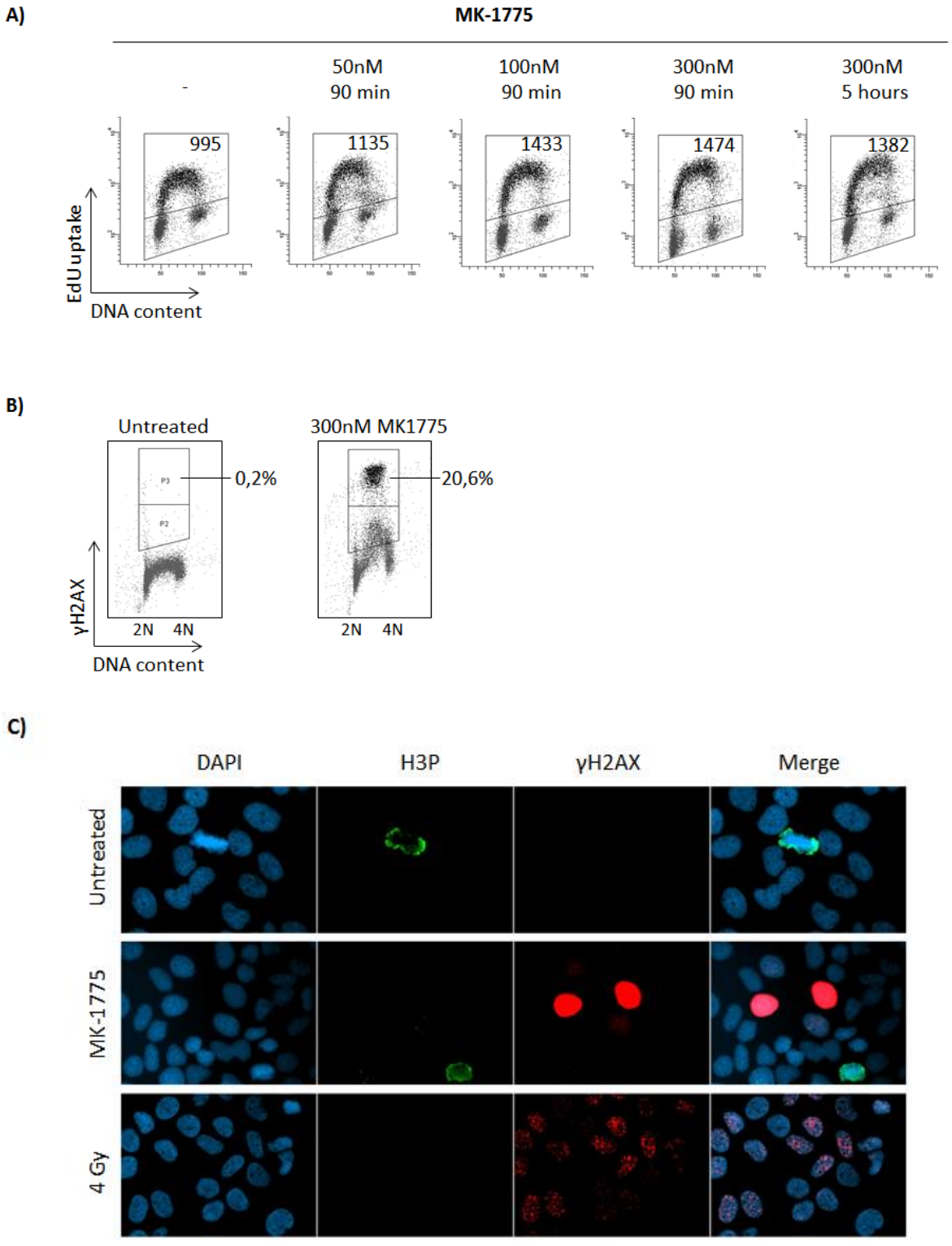

Figure10: S-phase effects of MK-1775. A) U2OS cells were treated with MK-1775 for 90 minutes or 5 hours. 1 hour before fixation they were given EdU to assess active replication. After fixation the cells were stained for EdU and analyzed by flow cytometry. The median values of EdU signals are indicated. B) Flow cytometric analysis of U2OS cells treated with 300nM MK-1775 for 20 hours and stained for γH2AX and the DNA stain Hoechst. Numbers indicate the percentage of cells in the top gate, showing strong γH2AX staining. C) Images taken by IF microscopy of U2OS cells given MK-1775 at a concentration of 100nM or irradiated with 4 Gy, fixed after 6 hours, and subsequently stained for H3P, γH2AX and the DNA stain DAPI. IR-exposure was performed to get a positive control of γH2AX staining.

5.1.3 Severe hypoxia leads to DNA damage signaling in S-phase

In addition to confirming some of the effects of WEE1-inhibition, we wanted to check some known effects of hypoxia. First of all, we wanted to see that hypoxic conditions in our hypoxia chamber could induce DNA damage signaling in S-phase, as described in previous studies (Hammond et al. 2002; Hammond et al. 2003; Hasvold et al. 2013). To do so, cells were put in the hypoxia chamber with an oxygen level of <0,03% O_2 for 20 hours before fixation. They were then stained for γH2AX and analyzed by flow cytometry. The hypoxic conditions did in fact induce γH2AX signaling (Figure 11, top row, middle graph). However, within 90 minutes after reoxygenation, the cells had lost most of these signals (Figure 11, top row, right graph). Cells exposed to hypoxia also activate a cell cycle arrest in G1-phase (Amellem et al. 1991) and this can be seen in the DNA histograms from this experiment (Figure 11, bottom row) where exposure to hypoxia has led to an accumulation of cells in G0/G1-phase.

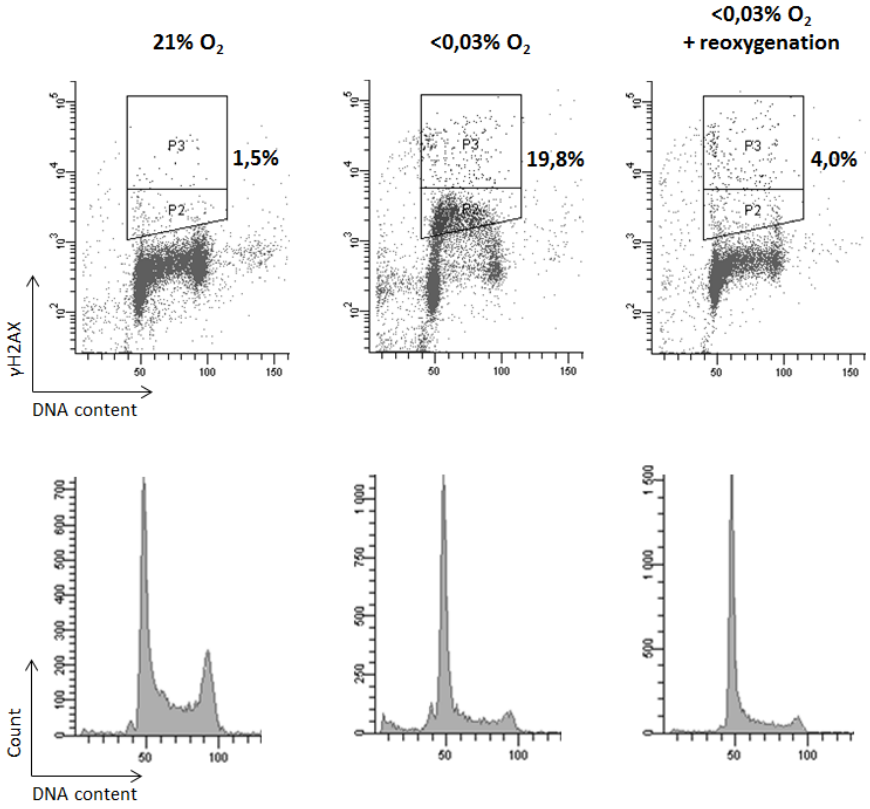

Figure 11: Hypoxia-induced DNA damage signaling and cell cycle arrest. Flow cytometric analysis of U2OS cells exposed to normal O_2-levels (21% O_2), hypoxia (<0,03% O_2) for 20 hours, and hypoxia (<0,03% O_2) for 20 hours followed by reoxygenation and subsequent incubation at 21% O_2 for 90 minutes, before fixation and staining for γH2AX and Hoechst. To investigate the effects of hypoxia, the cells were trypsinated inside the hypoxia chamber and exposed to a minimal amount of O_2 during fixation (middle row). The numbers in the top row indicate the total percentage of cells showing γH2AX signaling (the percentage of cells in the large gate).

5.2 MK-1775 efficiently inhibits CDK1 phosphorylation under hypoxia and after reoxygenation

Before starting to investigate the effects of hypoxia and reoxygenation on WEE1-inhibition, we had to affirm that the inhibitor we used, MK-1775, actually works during hypoxia and after reoxygenation, as hypoxia is known to alter the efficacy of many drugs (reviewed in Brown et al. 2004). To check that the inhibitor works during hypoxia, cells were grown in hypoxic conditions, and treated with MK-1775. Indeed, SDS-PAGE and Western blot analysis show that MK-1775 decreases phosphorylation of CDK1 (Y15) to a similar extent during hypoxia and normoxia (Figure 12A). To confirm that the inhibitor works after reoxygenation, cells were reoxygenated and subsequently treated with MK-1775. Also in this instance MK-1775 reduces the amount of CDK1 (Y15)-phosphorylation (Figure 12B, lane 4 compared to lane 3).

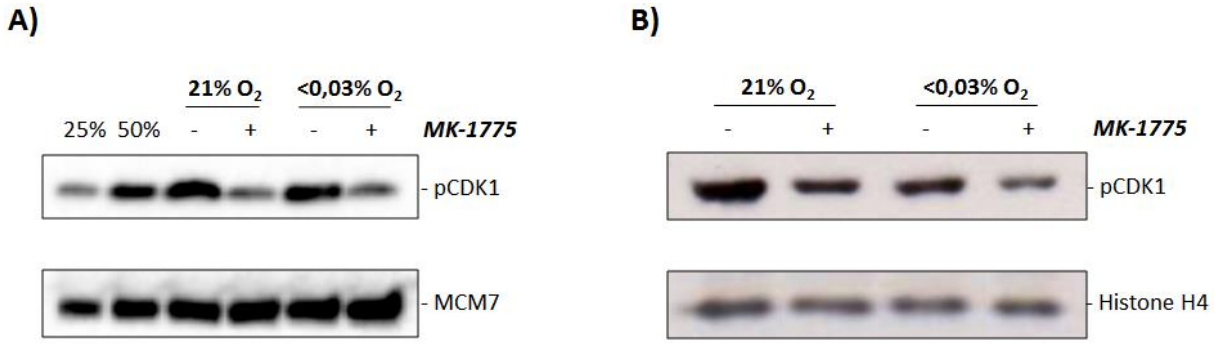

Figure 12: MK-1775 is working during hypoxic conditions and after reoxygenation. A) U2OS cells were kept in severe hypoxia (<0,03% O_2) for 4 hours, and treated with 100nM MK-1775 during the last hour. The samples were taken from the hypoxia chamber and directly frozen at -80°C, before lysis and protein analysis by SDS-PAGE and Western blotting. Cells given the same MK-1775 treatment in normoxic conditions (21% O_2) were used as a positive control for MK-1775 dependent reduction of CDK1(Y15) phosphorylation (pCDK1). MCM7 was used as the loading control. In lanes 1 and 2, 25% and 50% of the protein amount loaded in lane 3 (control sample) was loaded, respectively. B) U2OS cells were kept in severe hypoxia for 20 hours, treated with 100nM MK-1775 for 1 hour after reoxygenation, and analyzed as in A. Histone H4 was used as a loading control.

5.3 S-phase damage in response to MK-1775 treatment during hypoxic incubation

After confirming some of the basic cell responses to WEE1-inhibition and hypoxia, respectively, and having checked that MK-1775 inhibits CDK1 phosphorylation in hypoxic conditions, we moved on to study the impact of hypoxia on WEE1-inhibition. First, we explored S-phase damage during severe levels of hypoxia (<0,03% O_2). U2OS cells were treated with MK-1775 and placed in hypoxia (0,03% O_2) for 20 hours, followed by 90 minutes incubation at 21% O_2 before being fixed, stained for γH2AX and analyzed by flow cytometry. The 90 minutes incubation at 21% O_2 was included to allow dephosphorylation of γH2AX in the cells treated with hypoxia alone (Figure 11). A parallel set of samples were treated the same way with MK-1775, but kept under normoxic conditions. Figure 13A presents the dotplots from one such experiment, showing S-phase damage in response to MK-1775 for both normoxic and hypoxic samples. The mean percentage of cells in each of the gates (shown in Figure 13A) was calculated from three independent experiments, and the result is presented in the graphs in Figures 13B and C. They indicate that in general, there are no marked differences in the amount of S-phase damage between normoxic and hypoxic samples treated with MK-1775. However, the total percentage of cells positive for γH2AX in response to the highest concentration of MK-1775 (300nM) seems to be slightly higher in normoxic samples than in hypoxic samples (Figure 13B). The DNA histograms from the same experiment presented in Figure 13A are shown in Figure 13D. These (as well as the histograms from the other two experiments, not shown) indicate that there are somewhat fewer cells in S-phase in the hypoxic samples compared to the normoxic samples.

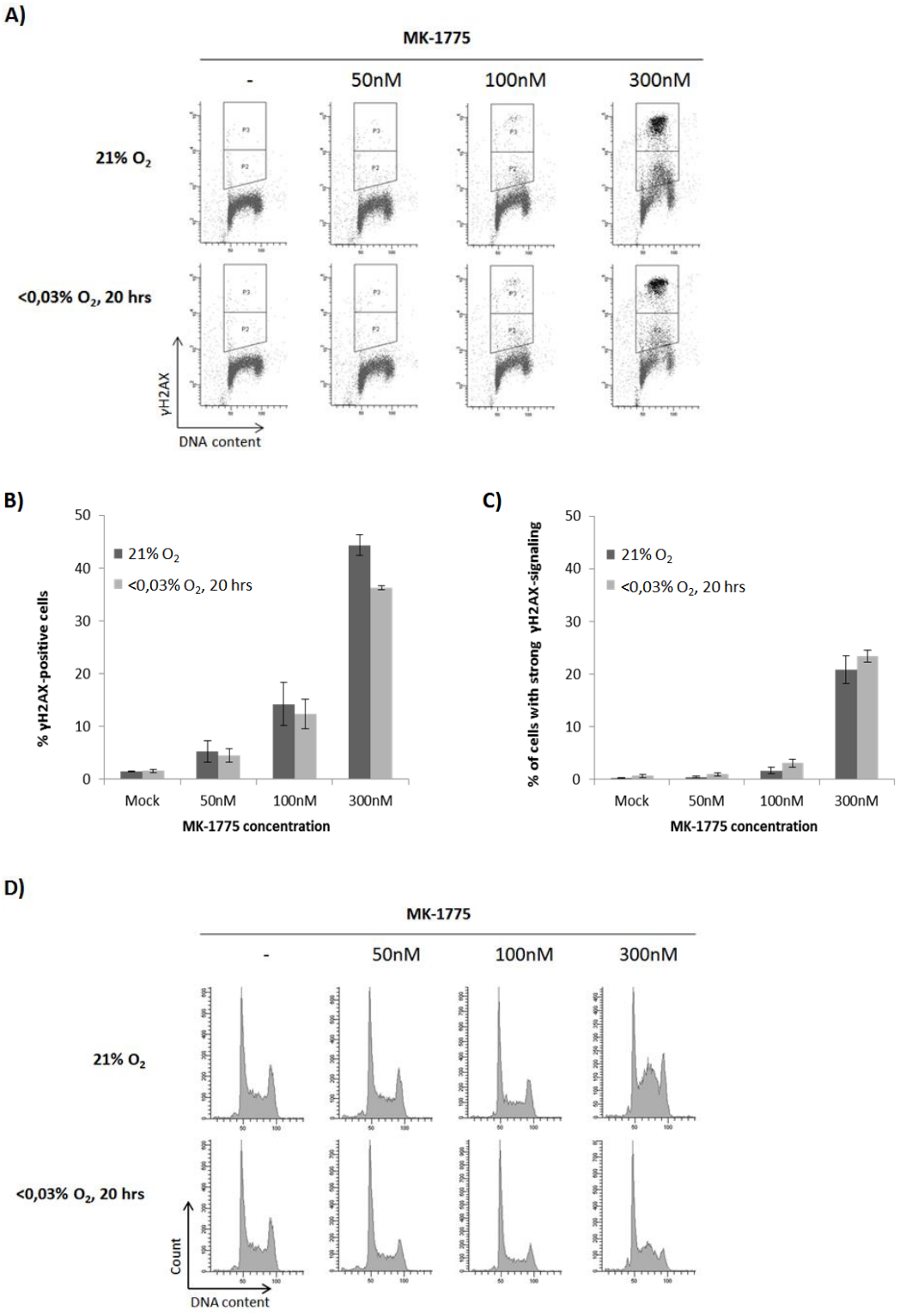

Figure 13: The influence of hypoxia on MK-1775 induced cell death and S-phase damage. A) Flow cytometric analysis of U2OS cells treated with MK-1775 for 20 hours during hypoxia (<0,03% O_2) and subsequently incubated for 90 minutes at 21% O_2, fixed and stained for γH2AX and Hoechst. In the graphs the gate for the total amount of γH2AX positive cells (large gate) and the gate for strong γH2AX signaling (top gate) are shown. B) Graph representing the percentage of cells positive for γH2AX, as shown in A). C) Graph representing the percentage of cells showing strong γH2AX signaling, as shown in A. D) Cell cycle profiles from the experiment shown in A. The numbers in B and C are mean values of 3experiments, and the error bars indicate SEM.

To test whether similar results were found under less severe hypoxia, the experiment was repeated with a moderate hypoxia level of 0,2% O_2 for 20 hours. Flow cytometric analysis of the samples show that the total percentage of cells positive for γH2AX in response to MK-1775 treatment is somewhat higher in normoxic samples than in hypoxic samples, whilst there does not seem to be a difference in cells having strong DNA damage signaling (Figure 14A). Since there were no big differences between the normoxic and hypoxic samples, we decided to explore whether longer exposures to hypoxia and MK-1775 treatments would give larger differences. MK-1775 was added before cells were placed in hypoxia (0,2% O_2) for 44 hours (Figure 14B) or 70 hours (Figure 14C). Also in these experiments, parallels were kept under normoxic conditions. The cells were then fixed and stained for γH2AX, and analyzed using flow cytometry. The results at 44 hours indicate a slight decrease in γH2AX-positive cells in hypoxic samples compared to normoxic samples, but no differences in high-level γH2AX signaling was seen (Figure 14B). At 70 hours there was no difference in the total percentages of γH2AX-positive cells between normoxic and hypoxic samples, though the fraction of cells showing strong γH2AX-signaling was slightly higher in the hypoxic samples (Figure 14C).

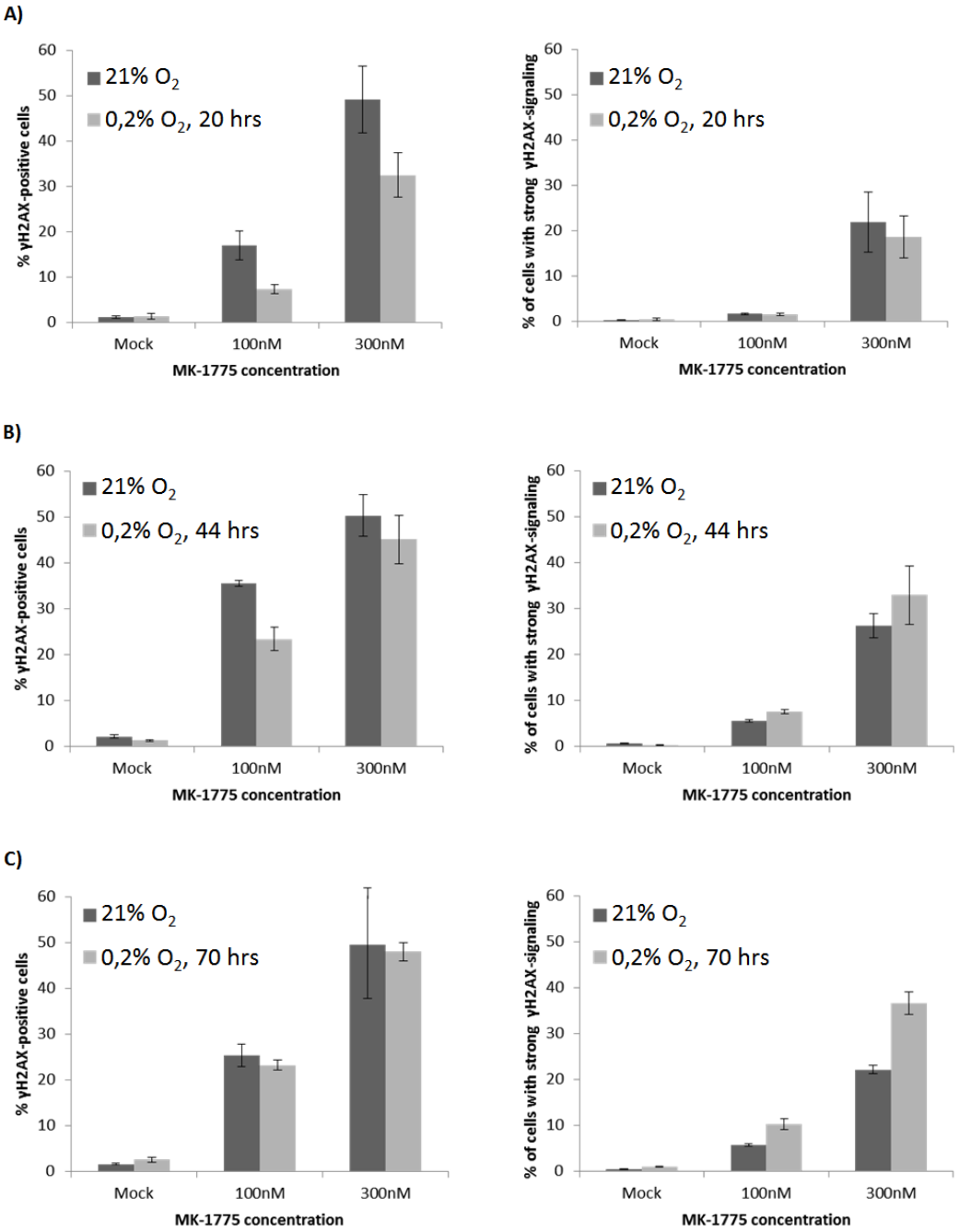

Figure 14: The influence on moderate hypoxia on the S-phase effects of MK-1775. A) U2OS cells were given MK-1775 and placed in hypoxia, 0,2% O_2, for 20 hours before fixation, staining for γH2AX and Hoechst, and analysis by flow cytometry. The percentage of cells positive for γH2AX (left graph) and strong γH2AX (right graph) was determined as in Figure 13. B) U2OS cells were given MK-1775 and placed in hypoxia, 0,2% O_2, for 44 hours before being analyzed as in A). C) U2OS cells were given MK-1775 and placed in hypoxia, 0,2% O_2, for 70 hours. All data in this figure are the mean values of 3 experiments, except for the bars marked with * which are the mean values of 2 experiments. Error bars indicate SEM.

5.4 S-phase damage in response to MK-1775 treatment after reoxygenation

Next, we wanted to investigate whether or not hypoxia followed by reoxygenation influences the S-phase effects of MK-1775. U2OS cells were first incubated at severe hypoxia (<0,03% O_2 for 20 hours), and thereafter reoxygenated and treated with MK-1775 at 21% O_2 for 24 hours. Again, parallels were kept under normoxic conditions, and the S-phase damage induced by MK-1775 was analyzed by γH2AX staining and flow cytometry. Figure 15A show dotplots of the results of one of these experiments. The mean percentage of cells in each of the gates (shown in Figure 15A) was calculated from three independent experiments, and the result is presented in the graphs in Figure 15B and C. These data indicate that there is no marked difference in S-phase damage between normoxic and reoxygenated cells treated with MK-1775. The DNA histograms from the same experiment as in Figure 15A are shown in Figure 15D. These profiles (and the histograms from the other two experiments, not shown) give no indication of a difference in the fraction of S-phase cells between normoxic and reoxygenated samples.

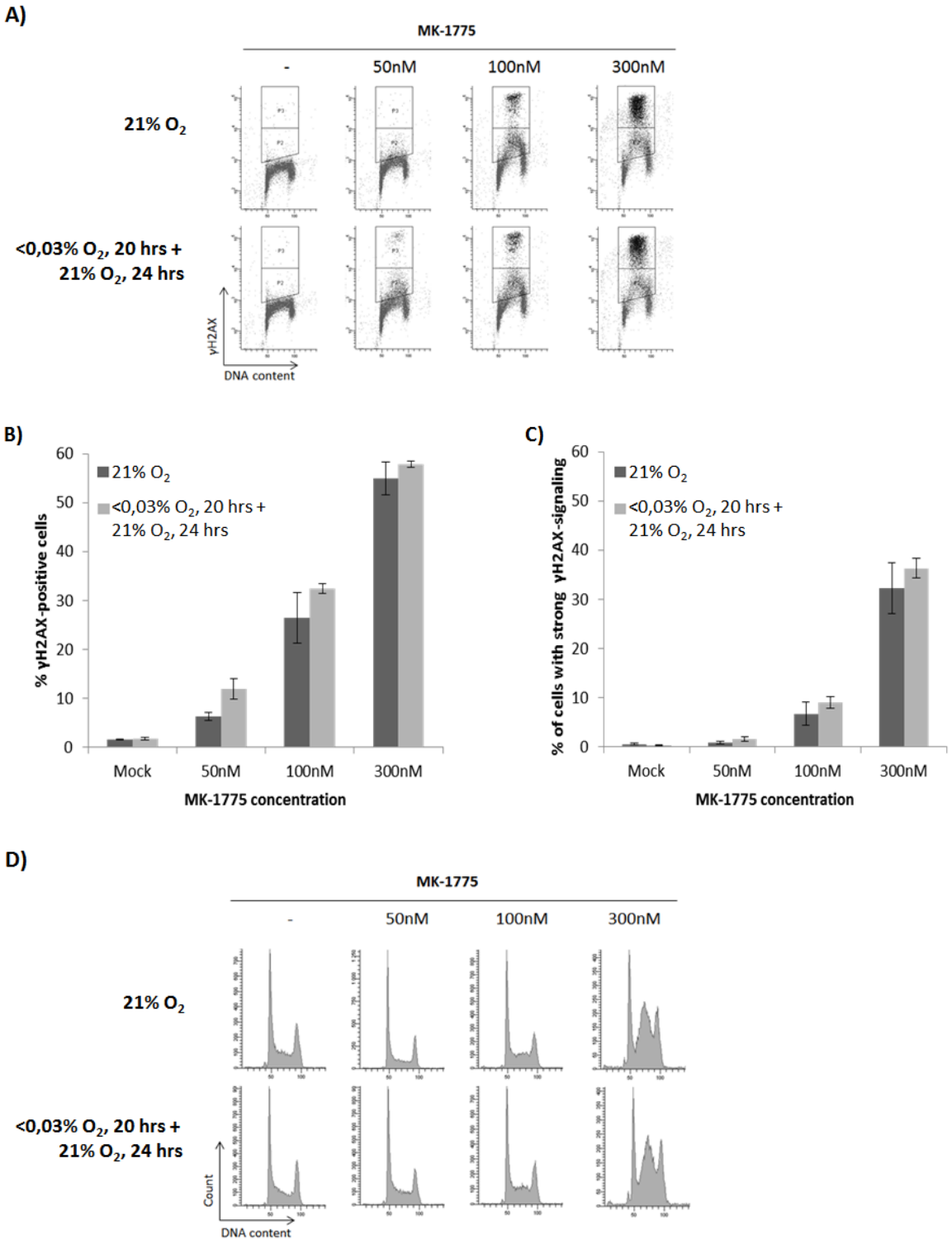

Figure 15: The influence of reoxygenation on MK-1775 induced cell death and S-phase damage. A) Flow cytometric analysis of U2OS cells treated with MK-1775 for 24 hours after reoxygenation following hypoxia (<0,03% O_2) for 20 hours. Cells were subsequently fixed and stained for γH2AX and Hoechst. In the graphs the gate for the total amount of γH2AX positive cells (the large gate) and the gate for strong γH2AX signaling (top gate) are shown. B) Graph representing the total percentage of cells positive for γH2AX). C) Graph representing the percentage of cells showing strong γH2AX signaling. D) Cell cycle profiles from the experiment shown in A. The numbers in B and C are mean values of 3 experiments, and the error bars indicate SEM.

Also in this case, we wanted to explore if similar results were found after moderate hypoxia. The cells were kept in 0,2% O_2 for 72 hours before MK-1775 was added for 24 hours. Flow cytometric analysis of γH2AX levels in these samples showed similar amounts of S-phase damage in the hypoxia-treated and normoxic cells (Figure 16).

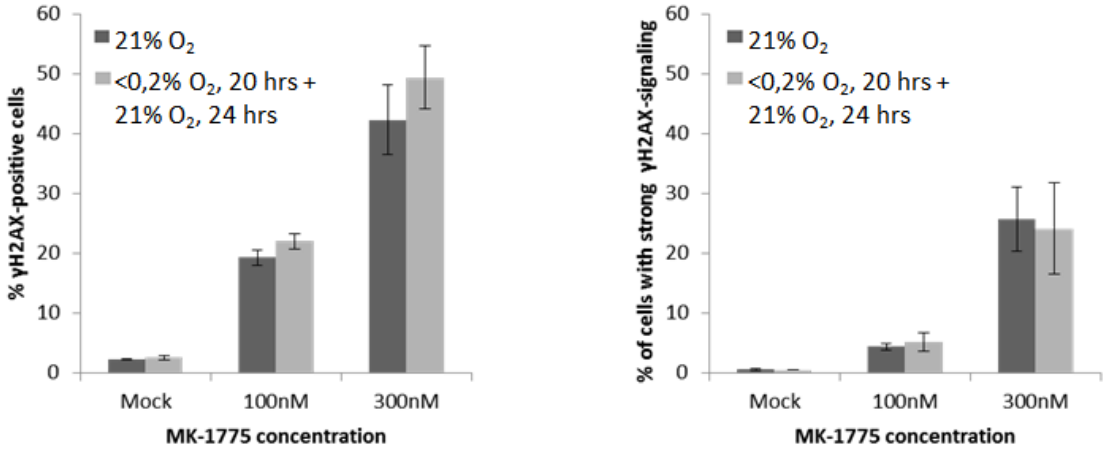

Figure 16: The influence of reoxygenation after moderate hypoxia on the S-phase effects of MK-1775. U2OS cells were placed in moderately hypoxic conditions (O,2% O_2) for 72 hours. Following reoxygenation, MK-1775 was added to the cells and they were kept for 24 hours in 21% O_2, after which the samples were fixed, stained for γH2AX and Hoechst, and analyzed by flow cytometry. A parallel set of samples were treated and analyzed the same way, but kept under normoxic conditions at all times. The percentage of cells positive for γH2AX in each sample was found by gating and the numbers were plotted in the left graph. The percentage of cells showing strong γH2AX signaling was also found by gating and plotted in the right graph. The bars represent the mean numbers from 3 experiments and error bars indicate SEM.

5.5 Hypoxia-induced alterations in expression of proteins that may affect the extent of S-phase damage

As described in the introduction (section 1.3.2) WEE1-inhibition causes S-phase damage through a cascade of events. Several proteins have been proposed to be involved in regulating these events, including MUS81, MRE11 and PLK1 (Dominguez-Kelly et al. 2011; Beck et al. 2012; Thompson et al. 2012; Szakal et al. 2013). In order to further explore how hypoxia may influence MK-1775-induced S-phase damage, we therefore wanted to measure whether or not hypoxia leads to altered expression of such proteins. To this end we have

performed two independent experiments where samples of U2OS cells were collected after hypoxic incubation as well as after reoxygenation, and the protein contents of the samples were analyzed by SDS-PAGE and Western blotting. In one of these experiments, the protein bands were imaged and protein levels quantified by using the BioRad ChemiDoc, and the results from this experiment are shown in Figure 17. When examining the blots in Figure 17A and the quantification numbers in Figure 17B, the levels of MUS81 stands out, as they seem to be increased by as much as 60% after reoxygenation. Also in the second experiment, where protein bands were detected by film and not quantified, MUS81 levels were increased after reoxygenation (data not shown). It is also worth mentioning the apparent decrease in RAD51 expression under hypoxia and after reoxygenation, which is consistent with previous reports showing that hypoxia causes decreased HRR (Bindra et al. 2004). It looks like hypoxia causes a slight decrease in PLK1 expression, but this might be underestimated due to the fact that the PLK1-antibody does not seem to bind in a linear manner (Figure 17A, lanes 1, 2 and 3).

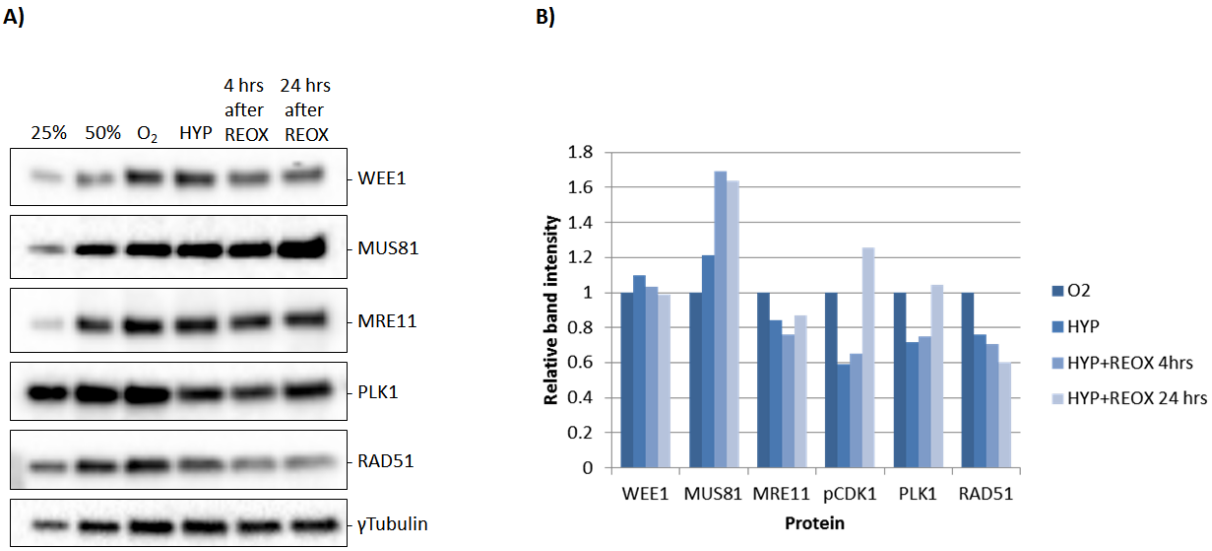

Figure 17: Hypoxia influences the expression of Mus81 and Rad51 and the phosphorylation of CDK1(Y15). U2OS cells were kept under severe hypoxic conditions (<0,03% O_2) for 20 hours, after which they were either directly frozen at -80°C or kept in 21% O_2 for 4 or 24 hours after reoxygenation before being frozen. A control sample was kept under 21% O_2 at all times. The protein contents were further analyzed by SDS-PAGE and Western blotting (A) and the levels of expression was assessed using the ChemiDoc camera and the ImageLab software (B). In A, 25% and 50% of the control sample (lane 3) was loaded in lanes 1 and 2, respectively. Results shown are from one experiment.

5.6 Measurements of cell survival

Furthermore, we wanted to examine whether hypoxia influences the overall cytotoxic effects of the WEE1-inhibitor. This was done by performing clonogenic survival assays. First, cells seeded at low densities were given MK-1775 at different concentrations before being placed in the hypoxia chamber for 20 hours or kept in 21% O_2 for the same amount of time. Afterwards, the inhibitor was removed and the cells were grown for 14 days before colonies were counted. The resulting survival curves shown in Figure 18A suggest that hypoxic cells are somewhat more resistant to MK-1775 induced cell death than cells kept in normoxic conditions.

Next, similar clonogenic survival assays were performed for cells treated with MK-1775 for 24 hours in 21% O_2 after reoxygenation (following 20 hours exposure to <0,03% O_2). The results presented in the survival curves in Figure 18B suggest that reoxygenated cells are slightly more sensitive towards MK-1775 than cells which have been kept under normoxic conditions. Thus, the efficacy of the WEE1-inhibitor appears somewhat increased if cells are treated with it after reoxygenation following prolonged hypoxia.

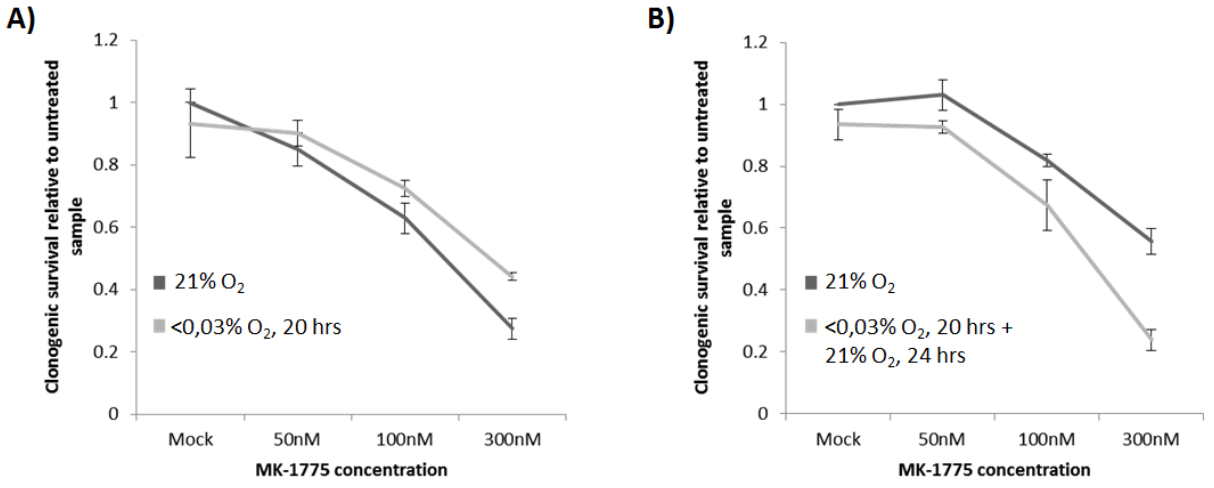

Figure 18: Cell survival after MK-1775 treatment during hypoxia and after reoxygenation. A) Survival of U2OS cells treated with MK-1775 for 20 hours during hypoxia (<0,03% O_2), after which the inhibitor was removed and cell colonies allowed to grow for 14 days before fixation. B) Survival of U2OS cells kept in severe hypoxia (<0,03% O_2) for 20 hours and treated with MK-1775 right after reoxygenation. The inhibitor was left on for 24 hours, after which the inhibitor was removed and cells allowed to grow for 14 days before fixation. The experiments were repeated 3 times and in all experiments there were triplicates for each sample condition. The error bars indicate SEM.

Finally, in attempt to explore why cells are dying after treatment with MK-1775, we performed preliminary immunofluorescence analysis of cells exposed to the inhibitor for 24 hours (Figure 19). The results clearly show γH2AX staining and nuclear abnormalities in response to the inhibitor, and it seems like some of the cells struggle to go through normal cytokinesis and are maintaining mitotic condensation (Figure 19, middle row).

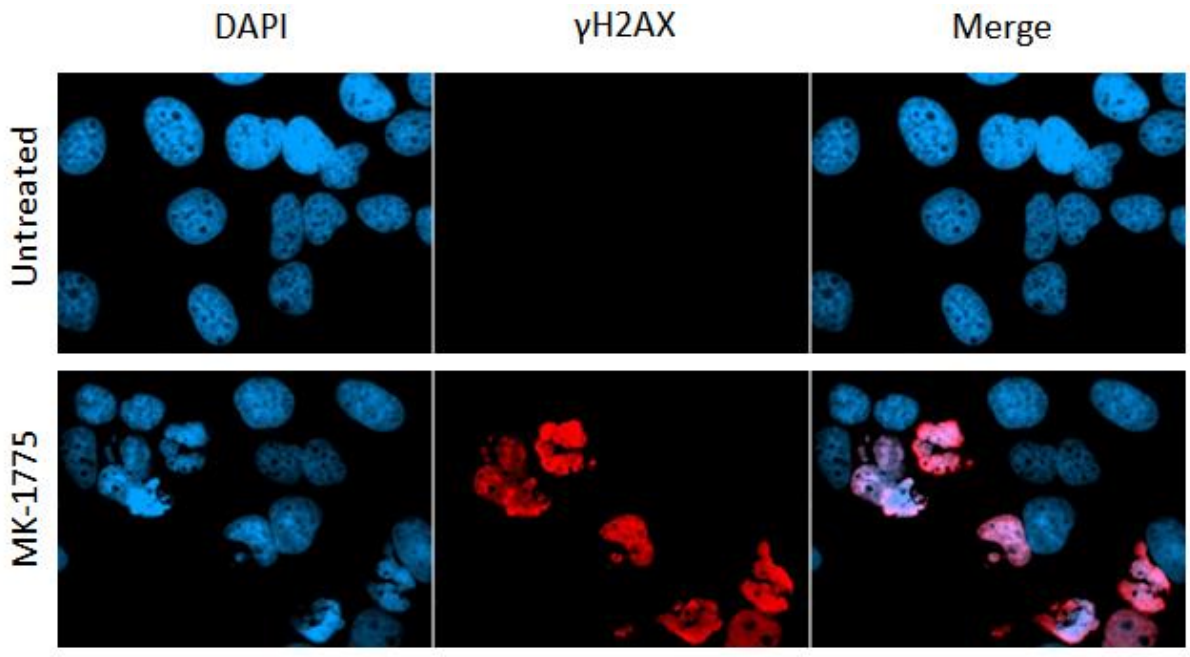

Figure 19: Immunofluorescence microscopy on MK-1775 treated cells show γH2AX signaling and nuclear abnormalities. U2OS cells were either untreated or treated with 300nM MK-1775 for 24 hours. Samples were stained with DAPI and γH2AX and viewed by fluorescence microscopy.

6 Discussion

6.1 General discussion

The purpose of this study was to investigate whether or not hypoxia influences the S-phase effects of the WEE1-inhibitor MK-1775. We did experiments with several concentrations of MK-1775 and different degrees of hypoxia, and showed that the MK-1775-induced γH2AX in S-phase is similar between U2OS cells kept at normal O_2-levels and cells exposed to hypoxia or hypoxia followed by reoxygenation. Comparing the levels of S-phase damage signaling to the degree of MK-1775-induced cell death suggests that S-phase damage likely contributes to cell death; however other effects of MK-1775 are also involved. Furthermore, we demonstrated that MK-1775 is toxic to hypoxic cells, and that reoxygenated cells are slightly more sensitive to the inhibitor than normoxic cells in terms of reduction in clonogenic survival. As MK-1775 currently is in clinical trials, these results could be of potential clinical importance.

6.2 The influence of hypoxia and reoxygenation on MK-1775-induced S-phase damage

We have investigated whether the S-phase effects of WEE1-inhibition are influenced by hypoxia and reoxygenation following hypoxia. We did not observe any marked influence of hypoxia on MK-1775-induced S-phase damage. Although multiple concentrations of MK-1775 were used and different hypoxic exposures were examined, the levels of S-phase damage were very similar between cells that were kept under normoxic conditions and cells that were exposed to hypoxia. This stands in contrast to a recently published study from our group investigating the influence of hypoxia on CHK1-inhibition, which showed that reoxygenated cells display more S-phase damage than normoxic cells after CHK1-inhibition (Hasvold et al. 2013).This may indicate that CHK1-inhibition causes S-phase damage through different mechanisms than WEE1-inhibition. This might be surprising as WEE1 and CHK1 have similar functions in the cell when it comes to control of CDK-activity, and both CHK1- and WEE1-inhibition result in increased CDK-activity, as well as increased origin firing and

DNA damage in S-phase (Syljuasen et al. 2005; Scorah et al. 2009; Beck et al. 2012). However, CHK1 has multiple targets involved in several different processes, as for instance DNA repair (Sorensen et al. 2005) and chromatin regulation (Groth et al. 2003), in addition to the inhibition of the CDC25 phosphatases that regulate CDK-activity (Sorensen et al. 2003). One possibility is therefore that additional CHK1 targets might be involved in suppressing S-phase damage. In contrast, few targets of WEE1 are known; CDK1 and CDK2 are well-known (Parker et al. 1992), and recently WEE1 was shown to also phosphorylate Histone H2B on Y32, an event involved in suppressing histone transcription (Mahajan et al. 2013). Consistent with CHK1 and WEE1 having at least partly separate functions important for DNA damage responses, synergistic cell killing has been observed after concomitant exposure to both CHK1- and WEE1-inhibition (Davies et al. 2011; Carrassa et al. 2012; Guertin et al. 2012).

As mentioned in the introduction, hypoxia is known to induce a G1-checkpoint causing a halt in cells entering S-phase (Amellem et al. 1991). This is also shown in the DNA histograms in Figure 11. Therefore, we had to take under consideration the fact that there might be fewer cells in S-phase in the hypoxic samples (see Figure 13D) and that this could influence our results if we were to calculate the percentages of γH2AX positive cells relative to the total number of S-phase cells. There is software (such as FlowJo 7.6) that can be used to analyze DNA histograms and calculate the amount of cells in each cell cycle phase (G0/G1, S or G2/M), and ideally we would have liked to do this for each experiment to find the levels of S-phase damage relative to the number of S-phase cells. However, this software was not available to us at present. Visual inspection of the DNA histograms in Figure 13D indicate that if taken relative to the number of S-phase cells, the hypoxic cells could show slightly more S-phase damage compared to the normoxic cells. However, the effect of hypoxia on cell cycle distribution is relatively minor in our studies, and would not alter our major conclusions.

Even though cells are released from the G1/S-checkpoint when reoxygenated (Amellem et al. 1996; Gardner et al. 2001), reoxygenation following hypoxia can affect cell cycle progression. For instance, it can induce a CHK2-dependent G2/M-checkpoint (Freiberg et al. 2006). Also, if a large population of cells is released from G1-arrest at the same time, the cells could be somewhat synchronized; hence the cell cycle profiles of reoxygenated

populations could look different than the cell cycle profiles of cells kept under normoxic conditions. By looking at the DNA histograms in Figure 15D however, there seems to be no noticeable differences in the cell cycle profiles of the normoxic versus the reoxygenated samples 24 hours after hypoxic exposure. To that note, we conclude that calculating the percentages of cells positive for γH2AX relative to the number of S-phase cells would also in this case be unlikely to cause very different results that the ones presented.

6.3 Does the MK-1775-induced S-phase damage lead to cell death?

As many previous studies examining WEE1-inhibition have focused on its ability to abrogate the G2/M-checkpoint and in this way induce cell death either when used alone or in combination with other genotoxic agents (see section 1.3.3), it would be interesting to study whether or not the S-phase damage caused by WEE1-inhibition also contributes to cell death. It has been shown that the phosphorylation of Histone H2AX (S139) is reversible in cells showing low levels of this phosphorylation, although these cells resume cell cycling with decreased replication rates (Nähse-Kumpf, manuscript in preparation). However, the phosphorylation is not reversible in cells showing strong γH2AX signals (the population in the top gate in Figures 8B, 13A and 15A). Furthermore, such populations with strong γH2AX signals are also TUNEL positive, indicating massive DNA breakage, and these cells are thought to subsequently die (Nähse-Kumpf, manuscript in preparation) as a result of them going into premature mitosis, which leads to mitotic catastrophe and/or apoptosis (Beck et al. 2010). Comparing the graph in Figure 13C with the survival data in Figure 18A shows that whilst approximately 20% of the cells show strong γH2AX signals in response to the highest concentration of MK-1775, as much as 60-70% of the cells die in response to the same inhibitor concentration. The same is seen in Figure 15C and 18B, where 30-40% of the cells show strong γH2AX signals whilst the surviving fraction is reduced by 40-75%. These numbers indicate that whilst the S-phase damage might very well contribute to MK-1775-induced cell death, there must be other effects of the inhibitor also involved.

6.4 Do MUS81 levels in hypoxic and reoxygenated cells correlate with MK-1775-induced S-phase damage?

Preliminary experiments were performed in order to assess if hypoxia and reoxygenation alter the levels of certain proteins thought to be involved in the events leading to DNA damage following WEE1-inhibition. The result showed a marked increase of MUS81 levels in reoxygenated cells compared to normoxic cells (Figure 17). MUS81 is an endonuclease involved in several processes in the cell (Hanada et al. 2007), and the DNA damage following WEE1-inhibition is thought to be a result of unscheduled MUS81-activity (Dominguez-Kelly et al. 2011; Beck et al. 2012) (see section 1.3.2). However, no marked increase in S-phase damage was seen when γH2AX levels in reoxygenated and normoxic cells were analyzed by flow cytometry (Figure 15B and C). This could indicate that it is not necessarily the protein levels that regulate MUS81-activity, but rather post-translational modifications. It would be interesting to explore the means of MUS81 activation in further studies. In yeast, MUS81-activity depends on polo-like-kinase Cdc5, (Gallo-Fernandez et al. 2012) which corresponds to PLK1 in humans. The increase in MUS81 levels might therefore potentially be related to the decreased PLK1 levels in hypoxia treated cells (Figure 17). One might speculate that MUS81 could be upregulated in attempt to counteract the decrease in PLK1 expression. Why MUS81 levels would be particularly increased after reoxygenation remains unknown, but it is likely linked to the fact that MUS81 is involved in replication restart (Hanada et al. 2007), which occurs when hypoxic cells are reoxygenated.

6.5 Potential clinical relevance of MK-1775-induced cell death in hypoxic and reoxygenated cells

Our clonogenic survival data suggest that MK-1775 is toxic when administered during hypoxic exposure (Figure 18A). It is almost as toxic in hypoxic cells as in normoxic cells, although hypoxic cells might be slightly less sensitive. This is probably a cell cycle effect; hypoxia causes a minor accumulation of cells in G1-phase, leading to somewhat fewer cells entering S-phase and G2-phase which are the phases where WEE1 and thus MK-1775 mainly acts. Our survival data also suggest that reoxygenated cells are slightly more sensitive

towards MK-1775 than normoxic cells (Figure 18B), which does correlate with results found for CHK1-inhibition in the aforementioned study (Hasvold et al. 2013). Altogether, provided that the inhibitor reaches the hypoxic cells in the tumor, our results clearly demonstrate that it would work and be toxic under both normoxic and hypoxic conditions. The fact that MK-1775 is effective and causes cell death in both normoxic and hypoxic cells is relevant to the clinic. MK-1775 is in phase I and phase II clinical trials both as a single agent and in combination with other chemotherapeutic drugs, being tested in patients with solid tumors (www.clinicaltrials.gov). As hypoxia is a common trait of solid tumors, and hypoxic cells often show resistance to chemotherapeutic drugs, it is of the essence to know whether or not a new drug is efficient in such cells. However, our results should be verified in several cell lines. Additionally, it is also important to keep in mind that findings *in vitro*, for instance results such as our survival data, would not necessarily be the same *in vivo* (see section 6.6.1).

Furthermore, it would be intriguing to elaborate a bit on the clinical relevance of our results as a new clinical trial is starting up in which MK-1775 is tested in combination with IR in patients with glioblastoma (www.clinicaltrials.gov). It has previously been shown that MK-1775 radiosensitized p53-defective cells (Bridges et al. 2011). When we did the preliminary IF microscopy experiment to look into the mode of cell death in MK-1775-treated cells (Figure 19), we also treated a sample with MK-1775 in combination with 4 Gy of IR, Interestingly, fewer of the cells treated with both IR and MK-1775 showed γH2AX signals and nuclear abnormalities than cells treated with MK-1775 alone. This experiment would of course have to be repeated to verify the results, but should the results be confirmed, it would suggest less S-phase damage after combined treatment with IR and MK-1775, compared to the S-phase damage after MK-1775 alone. Such findings would need to be taken into consideration as this effect might contribute to reducing the synergistic effect of the combined treatment.

Finally, it is important to consider the risks of using inhibitors of DNA damage signaling in cancer therapy. As mentioned in section 1.2.5, targeting of the DDR in cancer therapy is two-sided. On one hand, lost or inhibited DDR pathways might increase the vulnerability of cancer cells towards DNA damaging agents. On the other hand, losing components of the

DDR can lead to genetic instability which can drive cancer development. We have observed that MK-1775 induces DNA damage signaling; some cells show strong γH2AX signals and are thought to die at later time points, whilst other cells show weaker γH2AX signals and are thought to survive and resume cell cycling. So the question becomes; what happens to the cells that survive MK-1775 exposure? Could they become more genetically unstable and give rise to a more malignant phenotype? These questions enlighten the fact that targeting the DDR in cancer therapy is a balancing act, and more research on possible adverse effects of such treatments is needed. Whether or not WEE1-inhibition also has adverse effects when it comes to genetic instability and cancer progression remains unknown, but it would be interesting to do more experiments on this subject.

6.6 Experimental considerations

6.6.1 Cell culture

During this project, all experimental treatments have been performed on cultured human cells. There are many advantages with using cell lines in research; cultured cancer cells are relatively inexpensive as they can be frozen and kept for a considerable amount of time, and many methods for manipulating them are well established (for instance transfection). In addition, they are easy to work with, as adding chemical substances to the medium or subjecting the cells to irradiation are straight forward processes. There are also few ethical issues when using cultured cells compared to animal models. However, cell cultures do not directly reflect the conditions in a tumor, and cannot be used as a substitute for research in living organisms. The conditions the cells are kept under are very different from the environment in a tumor; especially regarding the oxygen levels (~21% O_2 in normal air and common incubators versus 2-9% O_2 in body tissues (Bertout et al. 2008), growth in a monolayer, and the fact that cells lose the interactions with and influences from the tumor microenvironment. Because most cancer cell lines are cultured for years, many of their genetic characteristics change considerably when the cells adapt to the new environment. It is important to keep this in mind, as findings made in cell cultures do not necessarily translate to living organisms. This is why evolving new chemotherapeutic drugs (or any other

drug for that sake) is a multistep process, starting in cell cultures, going via animal models to several rounds of clinical trials before the drug becomes available for patients. Nonetheless, cultured cells are essential as a model system used in basic research on biological mechanisms and as a tool for studying cancer cell responses to different treatments like chemotherapeutic drugs or radiation.

In this study we used the U2OS cell line as the model system. This cell line is well-known, and was chosen because much work on WEE1-inhibition has been done with U2OS cells both in our group and in other studies (Hirai et al. 2009; Beck et al. 2010; Hirai et al. 2010; De Witt Hamer et al. 2011; Dominguez-Kelly et al. 2011; Beck et al. 2012; Kreahling et al. 2012). It was therefore an advantage using this cell line in which the effects of inhibiting WEE1 were well studied, since we were looking to see if the cellular responses to WEE1-inhibition were altered when the cells were kept under hypoxic conditions. On the other hand, whether or not the cell has a wild-type p53 phenotype has significance for the cell responses to WEE1-inhibition (see section 1.3.3) and the U2OS cells do not have a fully functioning G1/S-checkpoint (Petersen et al. 2010) despite being wild-type for p53. Therefore, it would be interesting and necessary to do more research in other cancer cell lines, to verify if the same results are obtained. It would also be interesting to include normal cells in some of these studies, such as BJ fibroblasts, which are normal diploid cells.

6.6.2 WEE1-inhibition

To inhibit WEE1-activity we used the small molecule inhibitor MK-1775. We confirmed that MK-1775 causes a decrease in CDK1(Y15) phosphorylation within a short amount of time and that it works both under hypoxic conditions and after reoxygenation (see Figures 9A and 12). However, inhibitors in general can be prone to unspecific action on additional proteins. For instance, there is another inhibitor of WEE1 available; PD-166285 which has been used in other studies (Leijen et al. 2010). It could potentially be useful to use for verification of our results, but as it has been described as a broad-spectrum inhibitor of receptor tyrosine kinases (Panek et al. 1997), MK-1775 is a better choice for studying the specific effects of WEE1-inhibition. Because of such potential unspecific actions of inhibitors, interfering RNA techniques are sometimes used as they are more target-specific; although they are much more time consuming procedures. Based on this, it would be wise to also do experiments

using interfering RNA techniques for knocking down WEE1 in further studies, to confirm that the same cellular responses occur as with using MK-1775.

6.6.3 Hypoxia treatments

Central to this project was hypoxia, and the question of whether or not low O_2-levels influence the S-phase effects of MK-1775. We chose to perform most of our hypoxia-experiments at the most severe level of hypoxia that we could achieve (<0,03% O_2) because we expected that potential differences were most likely to occur when the O_2 levels were very low. However, some cellular responses vary with different levels of hypoxia. For instance, the hypoxia-dependent induction of γH2AX signaling previously described (Hammond et al. 2003; Hasvold et al. 2013) and also shown in Figure 11, is observed at prolonged exposures to severe levels of hypoxia (~0,03% O_2) but not at more moderate levels of hypoxia (0,2% O_2) (Hasvold et al. 2013). Because of these differences, we repeated some of the experiments at 0,2% O_2 (Figures 6 and 8). Furthermore, it could be interesting to also study the effects of MK-1775 during acute hypoxic exposure. Given that we did not discover major differences in MK-1775-induced S-phase damage between normoxic samples and samples exposed to low O_2 levels for a prolonged amount of time, it might be unlikely that we would see such differences after shorter time-frames of hypoxic exposures, but it would be worth looking into due to its physiological relevance.

A final point regarding the hypoxic treatment and the hypoxic conditions achieved in our lab appears when comparing our cellular responses to those observed in other studies. As mentioned, hypoxia-dependent induction of γH2AX signaling was detected during this project, and has been described several times previously. However, we did not see any high-level γH2AX signaling after reoxygenation (Figure 11). This result deviates from a previous study reporting that reoxygenation following hypoxia (0,02% O_2) causes actual DNA damage (Freiberg et al. 2006). Such reoxygenation-induced DNA damage was not detected during this project nor has it ever been detected in our laboratory (Hasvold et al. 2013). This could be due to differences in experimental setups and differences in how severe hypoxia is achieved, and it highlights the fact that we should keep in mind that cellular responses might vary because of small differences in experimental setup and/or O_2 availability.

6.6.4 Measuring protein levels and modifications by flow cytometry

Flow cytometry is the main technique used in this study. By applying DNA and antibody staining, this method can be used to study several cell parameters simultaneously. Additionally, flow cytometry makes it possible to obtain a large amount of information about one cell can in a few milliseconds (Shapiro 2003, page 17). This makes flow cytometry a very effective method, where thousands of cells can be analyzed in a short amount of time. In this project, it was essential that DNA dyes together with antibody staining made it possible to look at the level of protein phosphorylations relative to cell cycle phase. Also, the fact that for every sample at least 10000 cells were analyzed gives the results robustness. There are additional advantages with flow cytometry, including the possibility of analyzing subpopulations of cells in a sample by gating, and being able to go back and work with old data to get new information by changing or adding gates.

When performing the staining procedures we processed samples individually, which gives a risk of variations in the results as a consequence of small differences in the sample handling. The barcoding technique is a nice method for minimizing sample-to-sample variations, as the samples are given an identifying dye concentration before being combined and stained together. After the sample solution has been run through the flow cytometer, the individual samples can be separated on the basis of the identifying dye (Krutzik et al. 2006). Even though we did not discover any obvious issues with staining differences between samples in our experiments, barcoding could be an option for further studies. When we processed the data looking at differences in γH2AX-signaling between normoxic and hypoxic samples, we combined the results from three experiments performed at different times. We sought to minimize variations by making sure that all samples within one experiment got inhibitor and antibodies from the same batches, and by doing the procedures as similar as possible in all experiments to avoid differences in other factors that could cause variations in the results. For instance, the cells were plated at the same density in order to prevent them from growing too dense, which could influence factors like cell cycle progression and oxygen consumption. Another step to minimize variations between the experiments could be to normalize the data in each experiment relative to one sample. This could make deviations in the combined results smaller. However, in the results presented here we have combined the numbers from the experiments directly, without any normalization. We also did the

calculations with normalizations, but as the results were very much the same and the standard error numbers also were very similar, we chose to present the direct data because this is more straightforward and clear.

A limitation of flow cytometry is the limited number of antibodies available to combine with this technique. During this project, antibodies against WEE1, pCDK1 (Y15) or MUS81compatible with flow cytometry would have been very useful. But at this time no such antibodies were available, and we therefore had to rely on other methods such as Western blotting.

6.6.5 Measuring protein levels and modifications by Western blotting

Western blot analysis is, as flow cytometry, based on antibody staining. Aside from this, the methods are very different. As mentioned in the previous section, there are limitations in antibodies available for flow cytometry. For Western blotting on the other hand, most commercially available antibodies can be used. Additionally, more proteins and/or modifications can be measured during one procedure because the nitrocellulose membrane can be cut into pieces according to the sizes of the proteins of interest, and there are no limitations due to spectral overlap as there is with flow cytometry. However, during sample preparation for Western blotting, the protein contents of all cells in one sample are mixed and analyzed together. Therefore, the method is most suited for studying protein levels and modifications in total cell populations. Furthermore, it can be difficult to examine protein levels and modifications relative to cell cycle phase using Western blotting. It is possible by synchronizing cells or sorting them before the Western blot analysis, but these processes are more laborious and time-consuming than doing these forms of analyses by flow cytometry. Based on this, only experiments in which it was not essential to know the cell cycle phases of the cells, or where antibodies compatible with flow cytometry were not available, were performed by Western blot in this project.

Using Western blotting for quantification of differences in protein levels and modifications between samples requires taking several precautions. One of the precautions we took was doing protein concentration measurements of the samples before performing SDS-PAGE. By doing this one can load the appropriate amount of protein in each well of the gel and avoid saturation of antibodies on the membrane later. In addition, in most of the cases we loaded

25% and 50% of the control sample in order to check the linearity of the results. When comparing the band representing the proteins or protein modifications with the loading control, it is important to also look at this linearity to avoid misinterpretation of the results. In the one experiment from which we have presented quantification data of a Western blot analysis (Figure 17) we did load 25% and 50% of the control sample to get as reliable data as possible. In addition, the BioRad ChemiDoc was used, and this system gives more reliable results than the more conventional photographic film as has it a greater linear range and controls for signal saturation.

6.7 Concluding remarks

WEE1 is known as a checkpoint regulator and it has been shown that the WEE1-inhibitor MK-1775 induces checkpoint abrogation. This has been the basis for using MK-1775 to target checkpoint-deficient cancer cells in cancer therapy. However, WEE1 is also important for controlling CDK-activity during normal cell cycle progression, and WEE1-inhibition is shown to induce damage in S-phase cells. We believe that this damage also contributes to cell death following WEE1-inhibition, and this is a major reason for studying the S-phase effects of MK-1775. Additionally, studies of the S-phase effects of WEE1-inhibition can give more insight into the specific role of CDK regulation by WEE1 during normal cell cycle.

As hypoxia is a common trait of human tumors, and is known to affect both the DDR and the efficacy of different cancer treatments, it is of interest to investigate whether or not it has an impact on the effects of MK-1775. We have shown that the drug works and is toxic under hypoxic conditions and after cells have been reoxygenated. Furthermore, we have also shown that the S-phase damage following WEE1-inhibition is similar in cells kept under normoxic conditions and cells exposed to hypoxia. Also, this S-phase damage likely contributes to cell death, although other effects of WEE1-inhibition must also be important for this. Although these findings need to be confirmed in further experiments, they may be relevant to the clinic as MK-1775 is currently tested as an anti-cancer drug in several clinical trials.

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Sissel Hauge

8 List of abbreviations

ARF	Alternate reading frame
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related
ATRIP	ATR-interacting protein
BCL-2	B-cell lymphoma 2
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
CDC	Cell Division Cycle
CDK	Cyclin-dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CHK	Checkpoint kinase
CtIP	CtBP-interacting protein
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA damage response
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DSB	Double strand break
DTT	Dithiothreitol
ECL	Enhanced chemiluminescent
EDTA	Ethylenediaminetetraacetic acid
EdU	5-ethynyl-2´-deoxyuridine
FBS	Fetal bovine serum
H3P	Phospho-Histone H3
HIF1	Hypoxia-inducible factor 1
HRE	HIF-responsive element

HRP	Horseradish peroxidase
HRR	Homologous recombination repair
IC_{50}	Half maximal inhibitory concentration
IF	Immunofluorescence
IgG	Immunoglobulin G
INK4a	Cyclin-dependent kinase inhibitor 2A isoform p16INK4a
IR	Ionizing radiation
MCM	Mini-chromosome maintenance
MDM2	Mouse double minute 2 homolog
MMR	Mismatch repair
MRE11	Meiotic recombination 11 homolog A
MRN	MRE11-RAD51-NBS1
mTOR	Mammalian target of rapamycin
MUS81	MUS81 structure-specific endonuclease
NBS1	*Nijmegen* breakage syndrome protein 1
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
ORC	Origin recognition complex
PAGE	Polyacrylamide gel electrophoresis
PALB2	Partner and localizer of BRCA2
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate Buffer Saline
PCNA	Proliferating cell nuclear antigen
PIKK	PI3K-like kinase
PLK1	Polo-like kinase 1
pRB	Retinoblastoma protein
pre-RC	pre-Replicative complex
RAD51	DNA repair protein Rad51 homolog 1

RNA	Ribonucleic Acid
RNR	Ribonucleotide reductase
ROS	Reactive oxygen species
RPA	Replication protein A
SDS	Sodium dodecyl sulfate
SSB	Single strand break
ssDNA	Single stranded DNA
TrisHCL	Tris(hydroxymethyl)aminomethane hydrochloride
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UPR	Unfolded protein response
VHL	von Hippel-Lindau
WEE1	Wee1-like protein kinase
γH2AX	Gamma-Histone H2AX

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